The Chemistry and Evolution of Enzyme Function:
Isomerases as a Case Study

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This dissertation is the result of my own work and contains nothing which is the outcome of work done in collaboration except where specifically indicated in the text.

No part of this dissertation has been submitted or is currently being submitted for any other degree or diploma or other qualification.

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Cambridge, 31st July 2014

Sergio Martínez Cuesta
To my parents and my sister
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Abstract

The study of the evolution of proteins has been traditionally undertaken from a sequence and structural point of view. However any attempt to understand how protein function changes during evolution benefits from consistent definitions of function and robust approaches to quantitatively compare them. The function of enzymes is described as their ability to catalyse biochemical reactions according to the Enzyme Commission (EC). This dissertation explores aspects of the chemistry and evolution of a small class of enzymes catalysing geometrical and structural rearrangements between isomers, the isomerases (EC 5).

A comprehensive analysis of the overall chemistry of isomerase reactions based on bond changes, reaction centres and substrates and products revealed that isomerase reactions are chemically diverse and difficult to classify using a hierarchical system. Although racemases and epimerases (EC 5.1) and cis-trans isomerases (EC 5.2) are sensibly grouped according to changes of stereochemistry, the overall chemistry of intramolecular oxidoreductases (EC 5.3), intramolecular transferases (EC 5.4) and intramolecular lyases (EC 5.5) is challenging. The subclass “other isomerases” (EC 5.99) sits apart from other subclasses and exhibits great diversity. The current classification of isomerases in six subclasses reduces to two subclasses if the type of isomerism is considered. In addition, the separation of groups of isomerases sharing similar chemistry such as oxidosqualene cyclases and pseudouridine synthases from chemically complex sub-subclasses like intramolecular transferases acting on “other groups” (EC 5.4.99) might also improve the classification.

An overview of the evolution of isomerase function in superfamilies revealed three main findings. First, isomerases are more likely to evolve new functions in different EC primary classes, especially lyases (EC 4), rather than evolve to perform different isomerase reactions. Second, isomerases change their overall chemistry and conserve the structure of their substrates and products more often than conserving the chemistry and changing substrates and products. Last, the relationship between sequence and functional similarity suggests that correlations should be investigated on the basis of closely related enzymes.
Although previous research assumes a one-to-one relationship between EC number and biochemical reaction, almost one-third of all known EC numbers are linked to more than one biochemical reaction. This complexity was characterised for isomerase reactions and used to develop an approach to automatically explore it across the entire EC classification. Remarkably, about 30% of the EC numbers bearing more than one reaction are linked to different types of reactions, bearing key differences in catalysed bond changes. Several recommendations to improve the description of complex biochemical reaction data in the EC classification were proposed.

This dissertation explores enzymes from a functional perspective as an alternative to classical studies based on homology. This standpoint might prove useful to help to search for sequence candidates for orphan enzymes and in the design of enzymes with novel activities.
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Hello, my name is Bond, Change Bond.

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Take it EC!
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Enzymes are the catalysts of life. They accelerate biochemical reactions up to the rates at which biological processes take place in living organisms. The first enzyme to be discovered was amylase in 1833 (Payen & Persoz, 1833), which was originally isolated from barley and catalyses the conversion of starch into sugars. This discovery triggered the development of commercial breadmaking and brewing techniques as well as the development of fermentative enzymes at the beginning of the 20th century. Sumner isolated and crystallised urease in 1926, an enzyme that catalyses the hydrolysis of urea into ammonia and carbamate (Sumner, 1926). His original findings led to the demonstration that enzymes are actually proteins, a topic of controversy at the time. As a result, he was awarded the Nobel Prize in Chemistry in 1946. Almost forty years elapsed until the first three-dimensional structure of an enzyme was determined using X-ray diffraction techniques. In 1965, Phillips and coworkers obtained the structure of the hen egg-white lysozyme, which hydrolyses peptidoglycan in bacterial cell walls (Blake et al., 1965). During the 1960s, studies by Anfinsen on ribonuclease shed light on the dynamic nature of protein structure and served as a model to explore protein folding and the development of proteolytic methods. Subsequent research on the active site and catalytic mechanism of lysozyme, ribonuclease and other enzymes and further work on the stereochemistry of enzyme-catalyzed reactions by Cornforth and coworkers revealed mechanistic enzymology as a new scientific discipline.

In parallel, scientists started to capture enzymes performing function in their biochemical contexts, known as metabolic pathways. The idea of cataloguing and representing everything known about metabolism emerged from the original illustrations depicted by Krebs of the citric acid cycle (Krebs, 1940), which later translated into the elaboration of comprehensive wall charts of metabolic pathways (Reitz et al., 2004) that still exist today in many biochemistry labs. Current efforts to make metabolic data publicly available in online repositories such as KEGG are prevalent (Kanehisa et al., 2012). For instance, the ten enzymes present in the glycolysis pathway make the biochemical transformation
of glucose into pyruvate possible. First elucidated by Meyerhof and coworkers in 1940 (Kresge et al., 2005), this process meets essential demands such as the supply of adenosine triphosphate (ATP) and other key biosynthetic intermediates to many cellular activities (Bar-Even et al., 2012).

The ability of enzymes to perform biochemical catalysis has been traditionally described in textbooks with two main characteristics: acceleration of reaction rate and specificity (Fersht, 1999; Silverman, 2002). Whereas overwhelming evidence from kinetics studies has extensively supported the former over many decades, exceptions to the latter formalised into the concept of enzyme promiscuity, also known as the ability of enzymes to catalyse more than one biochemical reaction. This discovery has radically changed the way we understand enzymes and has implications across a broad range of scientific disciplines, from the evolution of enzyme function (Copley, 2003; Khersonsky & Tawfik, 2010; O’Brien & Herschlag, 1999) to the root of biotechnology and biocatalysis (Hult & Berglund, 2007; Nobeli et al., 2009).

In this chapter, basic facts about the chemistry and evolution of enzymes are first described. Then, existing approaches to explore the similarity of enzymes are reviewed. Next, our subject of study, the isomerases, which are a small class of enzymes catalysing geometrical and topological rearrangements between isomers, are introduced. Finally, the structure of the thesis is presented.

1.1 Chemistry of enzymes

As declared by Silverman, “enzymes are highly efficient organic chemists”. Over many decades, enzymologists have collected data on different aspects of the chemistry of enzymes and reported their findings in the primary literature: biochemical reactions, usage of cofactors, kinetic data and mechanistic interpretations (Silverman, 2002). With the aid of structural studies, researchers revealed that enzyme catalysis takes place in a buried pocket within the enzyme structure known as the active site. Experimental evidence from X-ray crystallography suggested a model to explain how this process takes place: the enzyme and substrates initially form a complex in the active site which may induce large conformational changes in their structures. First suggested by Linus Pauling, substrate binding is followed by transition state stabilisation. Enzymes decrease the activation energy of the reaction because they are complementary in shape and electrostatic properties to the rate-limiting transition state, which explains the vast rate acceleration compared to uncatalysed reactions (Pauling, 1946). One or more reaction intermediates are generated which then turn into products and are finally released from the active site.
As the substrate approaches the enzyme, molecular recognition takes place and the complex enzyme-substrate arises from interactions of the substrate with various amino acids in the active site. There are two types of interactions driving the complex formation: covalent interactions, which involve the sharing of electrons; and non-covalent interactions comprising electrostatic, dipole, hydrogen bonding, hydrophobic and van der Waals forces. Non-covalent interactions also contribute to enzyme catalysis by stabilising the transition state and destabilising the ground state. Although individually weak, they collectively make a strong interaction that is maximum when the transition state is formed. In addition, their reversibility also allows the product to be released from the enzyme active site (Silverman, 2002). Despite the vast research aimed at building basic principles of enzyme catalysis, we still know very little about certain aspects of this phenomenon. One of the areas that has attracted considerable attention in recent years is the connection between enzyme motions and catalysis (Hammes-Schiffer, 2013).

1.1.1 Catalytic sites, mechanisms and cofactors

The active site of an enzyme comprises about ten to twelve amino acids. About three or four of them, known as catalytic amino acids, are directly involved in the catalysis of the biochemical reaction and form the catalytic site (Gutteridge & Thornton, 2005). Their role is defined according to the specific chemical function they performed in the mechanism. In general, catalytic amino acids tend to be conserved, they show slight preference to be located in coil regions of secondary structure and they exhibit limited solvent accessibility (Bartlett et al., 2002). Histidine, cysteine and aspartate are the amino acids more often engaged in catalytic activities, whereas aliphatic amino acids such as alanine, leucine and glycine are rarely involved (Holliday et al., 2009). Their most common functions are transition state stabilisation, general acid/base (proton donor and acceptor) and nucleophilic covalent catalysis (Bartlett et al., 2002; Holliday et al., 2009). In multiple occasions residues act in combination forming catalytic units, for instance some residues may polarise the substrates or orientate other residues in order to maximise the probability that catalysis occurs (Gutteridge & Thornton, 2005).

Information extracted from the literature about catalytic residues is available in public resources such as the Catalytic Site Atlas (CSA) (Furnham et al., 2014). This repository provides curated annotations on the residues that are engaged in catalytic activity in enzyme structures deposited in the Protein Data Bank (PDB) (Berman et al., 2013). Several computational strategies integrate these annotations for the inference of catalytic residues using sequence-based approaches that exploit sequence conservation or more sophisticated algorithms employing three-dimensional templates of the atoms present in the catalytic site (Barker & Thornton, 2003; Nosrati & Houk, 2012). These techniques proved
to be useful in predicting enzyme function from sequence and structure data (Laskowski et al., 2005b). Alternatively, researchers have also built repositories of catalytic site information for specific groups of enzymes (Gariev & Varfolomeev, 2006).

Enzymes use several types of chemical strategies to convert substrates into products - these are called mechanisms. Holliday and coworkers adapted an extended version of Ingold’s classification of mechanisms (Holliday et al., 2007a) and used it to build a database of enzyme mechanisms called MACiE (Holliday et al., 2012). A comprehensive analysis of the database revealed that more than two-thirds of enzyme reactions rely on acid/base chemistry, especially proton transfer reactions (Holliday et al., 2007b). The second most common mechanism is nucleophilic catalysis such as substitutions, additions and eliminations, whereas electrophilic reactions are rare. The chemical bonds O–H, N–H and C–H are commonly cleaved and formed in enzyme reactions.

To gain further insight into the enzyme mechanism, biochemists perform kinetics experiments to measure two key parameters: the Michaelis-Menten constant ($K_M$) and the catalytic rate constant ($k_{\text{cat}}$) (Stitt & Gibon, 2014; Wittig et al., 2014). In general, enzyme reactions are described by Michaelis-Menten kinetics where $K_M$ captures the substrate binding affinity to the enzyme and links the reaction rate with the substrate concentration. Secondly, the catalytic rate constant captures the speed whereby the substrate turns into product in the enzyme active site (Cornish-Bowden, 2014). Studies comparing $k_{\text{cat}}$ with the rate constant of the uncatalysed reaction ($k_{\text{uncat}}$) strongly support the idea of acceleration of reaction rate achieved in enzyme reactions. Some enzymologists prefer combining these two parameters in a ratio known as the specificity constant ($k_{\text{cat}}/K_M$), which measures the enzyme’s ability to discriminate among competing substrates. Nevertheless when $k_{\text{uncat}}$ data are available, the so-called catalytic proficiency ($((k_{\text{cat}}/K_M)/k_{\text{uncat}})$) is also common in the literature (O’Brien & Herschlag, 1999).

The enzymatic catalysis of many biochemical reactions requires the presence of cofactors. A cofactor is an organic molecule or metal ion that binds to the active site and is essential for catalysis. The use of cofactors allows enzymes to expand the catalytic abilities achieved by the 20 naturally occurring amino acids (Holliday et al., 2007b), yet not all enzymes require a cofactor. Organic cofactors are mainly composed by nucleotides, amino acids and fatty acids substructures. For instance, glutathione consists of three amino acids glutamate, cystein and glycine. Although they chemically resemble other metabolites in the cell, cofactors are on average significantly more polar and slightly larger (Fischer et al., 2010).
1.1.2 Enzyme classification

During the 20th century, the nomenclature and classification of enzymes has constantly being under debate. In the early days, enzymes were given trivial names in order to uniquely identify and distinguish them from the rest. Although trivial names were chosen by groups of biochemists, multiple trivial names were sometimes given to the same enzyme by different groups, likewise different enzymes were named the same way, which led to confusing and ambiguous discussions (Cornish-Bowden, 2014). For example, NADPH dehydrogenase was first known as “NADPH diaphorase” and “old yellow enzyme” due to its ability to reduce various dyes, both trivial names still persist today (Daugherty et al., 2013; Savignon et al., 2012). Soon after, D-amino acid oxidase was designated as “new yellow enzyme” and distinction between both enzymes became even more difficult. The remarkable increase in the number of newly discovered enzymes led to the development of a system to name and classify them in a consistent manner. Since 1956, the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) has been the committee responsible to name old enzymes according to which new enzymes could be classified. In an attempt to develop a unified system, the Enzyme Commission (EC) classification was created and enzymes are now named and identified systematically with an EC number; this code is a four-level description that is used to classify enzymes depending on the overall chemical transformation of substrates into products (Tipton & Boyce, 2000). The first level corresponds to six different classes according to the type of chemistry being carried out. Oxidoreductases catalyse oxidation/reduction reactions (EC 1), transferases transfer a chemical group (EC 2), for example, a methyl or glycosyl moiety from one compound to another; hydrolases perform hydrolysis of chemical bonds (EC 3), lyases also cleave chemical bonds by other means than by oxidation or hydrolysis (EC 4), isomerases catalyse geometric and structural changes between isomers (EC 5) and lastly, ligases join two compounds with associated hydrolysis of a nucleoside triphosphate molecule (EC 6). These EC classes are further divided in subclasses and sub-subclasses (second and third level, respectively) in line with a variety of criteria such as the chemical bond cleaved or formed, the reaction centre, the transferred chemical group and the cofactor used for catalysis. The final level of classification defines substrate specificity. For example, alanine racemase is identified as EC 5.1.1.1 and the four digits indicate the following: EC 5 refers to isomerases, EC 5.1 are racemases and epimerases, EC 5.1.1 are racemases and epimerases acting on amino acids or derivatives and finally, EC 5.1.1.1 indicates racemisation of alanine as substrate.

One of the goals of biochemistry and enzymology is to discover the molecular function of enzymes. Although strictly speaking EC numbers do not refer to enzymes but to reactions catalysed by enzymes, the assignment of EC numbers to enzymes is now a common routine
in the functional annotation of proteins and protein-coding genes in databases such as UniprotKB (The Uniprot Consortium, 2013) and Ensembl (Kersey et al., 2014). The EC classification serves as a bridge between biochemistry and genomics (Kotera et al., 2004) and has proved to be very useful. In this dissertation, we have explored alternatives to improve the description of biochemical reactions in the EC classification following recent developments on the automatic search and comparison of enzyme reactions based on chemical attributes (Rahman et al., 2014).

1.2 Evolution of enzyme function

The ability of organisms to adapt to the changing conditions of their habitat is crucial to guarantee their survival and reproduction. For example, the capability of bacteria to acquire resistance for drugs and pesticides. At the metabolic level, this process of adaptation is related to the ability of enzymes to evolve beneficial functions in an environment of changing chemical conditions (Copley, 2012). Multiple research studies have unveiled the genetic mechanisms whereby innovation in enzyme function emerges. In its simplest form, the genetic diversity driving adaptation relies on the accumulation of point mutations. Although the majority of them are neutral or deleterious, gain-of-function mutations may create a new promiscuous activity in an existing enzyme. Subsequently, beneficial mutations might either increase the level of the promiscuous activity or when occurring in regulatory regions, they might enhance gene expression and therefore boost the cellular concentration of the enzyme up to physiological levels (Schulenburg & Miller, 2014). If the new activity provides a selective advantage to the organism in its ability to perform a fundamental biological process such as the competition for resources, beneficial mutations that enhance this activity will gradually strengthen the fitness of the organism. Then the function might either remain as a promiscuous activity of a multifunctional enzyme or segregate as the primary activity of a new enzyme through evolutionary processes such as gene duplication and specialisation (Glasner et al., 2006b). These observations are captured in a model known as Innovation-Amplification-Divergence (IAD) (Copley, 2012), which is considered to be iterative so the newly evolved enzyme may develop new promiscuous functions leading to further adaptive cycles (Kaltenbach & Tokuriki, 2014).

In this context, the evolution of new enzyme functions from the repertoire of existing activities plays a fundamental role on how organisms are able to perform adaptation. A model revealing promiscuous enzymes as evolutionary intermediates has been recently presented where the process of functional innovation is explained as a transition from specialised to promiscuous enzymes and vice versa (Khersonsky & Tawfik, 2010). Evidence from directed evolution experiments showed how mutations can enhance a promiscuous activity while maintaining the primary activity almost intact (Aharoni et al., 2005).
Promiscuous activities are usually more plastic to mutations than primary ones, which are robust because they have been longer under selective pressure. From a structural perspective, the acquisition of new enzyme functions is currently explained by stability-function trade-offs (Socha & Tokuriki, 2013). A recent study showed that an enzyme having an active site hard-wired to the structural scaffold is less likely to evolve new functionality than enzyme bearing an active site with low density of contacts and good separation from the scaffold. The latter scenario facilitates the evolution of new enzyme functions (Dellus-Gur et al., 2013).

Previous studies focusing on analysing enzyme superfamilies (Gerlt & Babbitt, 2001; Todd et al., 2001) and directed evolution experiments (Khersonsky & Tawfik, 2010) discovered aspects of how the evolution of enzyme function is influenced by aspects of the chemistry of enzymes. The overall chemical reaction is often changed by recruiting different catalytic residues within an active site, whilst conserving a few residues required for the catalysis of at least one mechanistic step of the overall reaction (Bartlett et al., 2003; Galperin & Koonin, 2012). Similarly, binding different substrates is commonly achieved by changing the residues involved in substrate binding and conserving residues involved in the overall reaction (Nobeli et al., 2005). There is substantial evidence supporting changes of the overall chemical reaction (Gerlt et al., 2012), as well as results reporting the importance of binding different substrates in the evolution of function in superfamilies (Furnham et al., 2012a). Commonly, enzyme superfamilies evolve by a combination of these two strategies: chemistry-driven and substrate-binding-driven evolution (Babbitt, 2003; Chiang et al., 2008). For instance, phosphate binding sites are often conserved, whilst the rest of the substrate can be changed during evolution (Amyes & Richard, 2013; Khersonsky et al., 2011).

Other comprehensive studies on the variation of enzyme sequence and structure (Meng & Babbitt, 2011; Pandya et al., 2013) and plasticity of active sites (Dessailly et al., 2013; Todd et al., 2002) have also been fundamental in understanding how homologous enzymes accommodate alternative chemistries. Similarly, research on the convergent evolution of enzyme mechanisms (Almonacid et al., 2010) and active sites (Gherardini et al., 2007) presented nature’s strategies to evolve different structural solutions for the catalysis of similar reactions (Almonacid & Babbitt, 2011; Elias & Tawfik, 2012; Galperin & Koonin, 2012). The widespread interest in understanding the evolution and chemistry of enzymes has led to large-scale collaborative projects such as the Enzyme Function Initiative (EFI) (Gerlt et al., 2011) which aims to determine enzyme function using both experimental and computational approaches. Starting from a comprehensive alignment of genomic regions, Zhao and co-workers from the EFI have identified the epimerase activity, pathway context and biological role in osmoprotection of a structurally characterised enzyme of
unknown function from *Pelagibaca bermudensis* using a combination of virtual screening, metabolomics, transcriptomics and biochemical experiments (Zhao et al., 2013).

## 1.3 Similarity between enzymes

Most studies exploring the similarity between enzymes share a common theme. They assume some form of correlation between protein sequence and function (Friedberg, 2006). Although the limits of this assumption have been explored extensively, our ability to investigate this correlation is limited by the available methods that measure similarity between enzymes and the quality of the experimental annotations in enzyme data. Researchers compare enzyme sequences and structures in order to extract relationships of common ancestry (homology), which arise due to speciation (orthology) or duplication events (paralogy) (Gabaldón & Koonin, 2013). Orthologous enzymes catalyse the same biochemical reaction in different species and their accurate identification is considered a crucial step in the automatic assignment of enzyme function in newly sequenced genomes. These strategies are useful to understand how enzymes evolve, however the advent of computational approaches to measure similarity between enzymes based on the genomic context, biochemical reactions and mechanisms adds a new dimension to existing evolutionary studies based on sequences and structures.

### 1.3.1 Comparing sequences and structures

In general, enzymes are regarded as similar if they are evolutionary related as measured by comparing their sequences and structures. During evolution, mutations occurring in enzyme sequences translate into changes in their three-dimensional structures. Early studies presented comparative analyses of the sequences and structures of homologous enzymes (Chothia & Lesk, 1986). The emergence of protein databases (Berman et al., 2013; The Uniprot Consortium, 2013) and tools to perform sequence comparisons (Altschul et al., 1990) allowed researchers to define “average” sequence similarity thresholds for the accurate transfer of function between enzymes using homology (Rost, 2002; Tian & Skolnick, 2003; Todd et al., 2001) and subsequent efforts integrated structural data (Laskowski et al., 2005b) and catalytic residue conservation (George et al., 2005) to help to refine this assignment. Related enzymes are nowadays grouped in families (Punta et al., 2012) and superfamilies (Andreeva et al., 2014; Sillitoe et al., 2013) according to sequence analyses and structural similarity, respectively. These resources rely on both the manual and automatic identification of domains, which are structurally and functionally conserved regions, also evolutionary building blocks within proteins. Whereas small enzymes contain only one domain, medium and large enzymes contain two or more domains, which are arranged in sequence defining a multidomain architecture (MDA) (Tamuri & Laskowski,
Evolutionary processes such as gene duplication and divergence generate different combinations of domains allowing enzymes to acquire new functionality (Bashton & Chothia, 2007). The recent explosion of sequence information in biological databases triggered the improvement of techniques that use Hidden Markov Models (HMMs) to capture fine details of the conservation of sequence and structural elements in protein domains (Eddy, 2011; Remmert et al., 2012). The availability of domain relationships in public resources has allowed researchers to explore the evolution of enzyme function on a superfamily basis (Baier & Tokuriki, 2014; Huang et al., 2012; Voordeckers et al., 2012) or across multiple superfamilies (Furnham et al., 2012a). For example, Furnham and colleagues explored the evolution of function across 276 enzyme superfamilies using FunTree (Furnham et al., 2012b), a resource containing phylogenetic trees decorated with structural, functional and mechanistic information representing the evolution of enzymes in a superfamily.

Multiple approaches aim to predict the likely function of enzymes of known sequence or structure but unknown function or mechanism. These employ several sources of evidence: sequence or structure similarity (Claudel-Renard et al., 2003; Desai et al., 2011; Quester & Schomburg, 2011; Yu et al., 2009), integration of conserved motifs, domains, catalytic and function determining residues (De Ferrari et al., 2012; De Ferrari & Mitchell, 2014; Kumar & Skolnick, 2012; Laskowski et al., 2005a; Nagao et al., 2014) and other features such as amino acid composition, secondary structure content and various physicochemical properties (Bray et al., 2009; Dobson & Doig, 2005; Kumar & Choudhary, 2012).

### 1.3.2 Comparing genomic context

Although sequence and structure methods are prevalent in evolutionary studies, the assignment of function based solely on this information is often unreliable due to the functional divergence between distant homologues in the same superfamily and functional convergence between unrelated enzymes (Omelchenko et al., 2010). In some cases, homology methods have been shown to produce incorrect functional annotations in protein databases (Hsiao et al., 2010; Schnoes et al., 2009) and do not always work well in the reconstruction of metabolic pathways (Karp, 2004). In order to overcome this, an array of strategies borrowed from comparative genomics integrate several sources of evidence from genomic context and provide an alternative to sequence and structure approaches (Plata et al., 2012). These strategies are useful to improve genome-scale metabolic reconstructions by filling metabolic gaps or missing network content (Green & Karp, 2007; Hanson et al., 2010) and to predict sequences for orphan metabolic activities (Kharchenko et al., 2006; Shearer et al., 2014; Smith et al., 2012; Yamada et al., 2012; Yamanishi et al., 2007). Researchers have discovered four main types of relationships shared by genes that
encode for enzymes belonging to the same metabolic pathway.

- They are more likely to be in close physical proximity on the chromosome. This phenomenon is more common in prokaryotes due to the existence of operon structures and regions transcribed by the same promoter (chromosomal gene clustering).
- They often share the same regulatory patterns, which lead to similar levels of gene expression (gene co-expression).
- They are either all present or all absent in an organism (phylogenetic profiles).
- They sometimes undergo fusion events, which translate into multifunctional enzymes or protein complexes acting on the same metabolic pathway (gene fusion).

The power of these approaches depends on the appropriate combination of these sources of evidence in order to find functional associations between the gene of unknown function and a database of genes. This strategy might help to prioritise the experimental tests aimed at discovering missing biological activities (El Yacoubi & de Crécy-Lagard, 2014). Multiple resources have been developed to accomplish this goal. For example, STRING is a database of functional associations between genes derived from genomic context, protein-protein interactions and literature. These are pre-computed and updated according to new releases of the source databases (Franceschini et al., 2013). Several efforts to extend genomic context associations with chemical information are currently in progress (May et al., 2013; Yamanishi et al., 2005).

1.3.3 Comparing biochemical reactions and mechanisms

During the rise of chemoinformatics as a scientific discipline, the idea to quantitatively compare small molecules developed into the advent of metrics to estimate chemical similarity. This concept proved useful in research areas such as drug discovery and chemical retrieval systems and by extension, the first approaches to measure enzyme similarity on the basis of their catalysed reactions first relied upon comparing their ligands directly (Chiang et al., 2008; Izrailev & Farnum, 2004; Nobeli et al., 2003, 2005). However enzyme reactions involve several molecules: substrates and products (reactants), therefore methods had to be extended in order to account for the transformation between two or more molecules. In a similar way that methodologies to calculate similarity between protein sequences and structures are necessary to understand protein evolution, finding similar enzyme reactions is a valuable tool for biochemists working in areas as diverse as chemical synthesis, enzyme reaction databases, enzyme design, metabolic network reconstruction and overall, discerning evolutionary relationships between enzyme sequence, structure and function.
Methods to measure similarity between biochemical reactions first relied on the structure of the EC classification system (Tohsato et al., 2000), however current approaches were extended to include chemical attributes (Bawden, 1991; Latino & Aires-de Sousa, 2011; Rose & Gasteiger, 1994). First, some strategies depend solely on the structure of the reactants and involve changes in the overall reaction, for instance bond changes and reaction centres. Second, alternative strategies based on catalytic mechanisms use mechanistic information derived from biochemical and structural studies. Although the mechanistic approach to compare biochemical reactions describes the process of enzyme catalysis in a more comprehensive manner (Almonacid & Babbitt, 2011), the amount of mechanistic information available in the literature and databases is not comparable to the extent of overall reaction data (Kraut et al., 2013).

Common approaches based on changes in the overall reaction are divided in two classes depending on whether atom-atom mapping (AAM), the one-to-one correspondence between the substrate and product atoms, is applied (Chen et al., 2013; Warr, 2014). First, approaches using AAM encode reactants in different ways. Gasteiger and coworkers calculated physicochemical and topological properties on the atoms and bonds present in manually-defined reaction centres in order to perform reaction similarity and classification (Hu et al., 2010; Sacher et al., 2009). A similar strategy was proposed whereby reaction centres are also manually-defined and classified in reaction classes depending on the functional groups undergoing transformation (Mu et al., 2011, 2006). Alternatively, a strategy known as Condensed Graph of Reaction (CGR) uses AAM to superpose atoms in substrates and products into a single pseudomolecule, which is then described in terms of substructure descriptors (de Luca et al., 2012). Some commercial algorithms are also common, for instance the CLASSIFY algorithm developed by InfoChem uses AAM to create circular environments around the atoms present in the reaction centre and serves as a search engine for similar reactions in organic chemistry resources such as SciFinder (Kraut et al., 2013).

The second group of approaches do not rely on AAM, yet reactants are also represented in similar ways as in the AAM strategies. Some approaches use physicochemical and topological properties to capture differences between the structures of substrates and products (Latino & Aires-de Sousa, 2006; Latino et al., 2008). Researchers associated with KEGG database (Kanehisa et al., 2012) developed a method to automatically detect the reaction centre using structure comparisons between reactant pairs and define reaction patterns to measure chemical similarity between biochemical reactions (Kotera et al., 2004). In a later study, they used these patterns to predict EC sub-subclasses (Yamanishi et al., 2009). Other strategies encoded biochemical reactions as differences between the molecular signatures (Faulon et al., 2008; Hu et al., 2012) or $^1$H NMR spectra
The existing methods proved useful in the development of databases of metabolites and biochemical reactions (Kanehisa et al., 2012), databases of substructures and functional groups (Kotera et al., 2008), to evaluate the performance of AAM algorithms (Muller et al., 2012), to discover inconsistencies in the EC classification (Apostolakis et al., 2008; Egelhofer et al., 2010; Kotera et al., 2004; Latino & Aires-de Sousa, 2009), to search and predict metabolic pathways (Hatzimanikakis et al., 2005; Moriya et al., 2010; Oh et al., 2007; Tohsato & Nishimura, 2009) and finally, to assign standard Gibbs energy changes ($\Delta G^0$) to thermodynamically uncharacterised biochemical reactions (Rother et al., 2010). However, the existing approaches have limitations. First, the high level of abstraction in the representation of reaction chemistry makes similarity results difficult to interpret. Second, the inability to detect changes of stereochemistry (chiral inversions and cis/trans isomerisations) in biochemical reactions was also a major disadvantage (Apostolakis et al., 2008). The EC-BLAST algorithm (Rahman et al., 2014) aims to overcome these drawbacks. It extracts bond changes and reaction centres using electron shift patterns as calculated using the Dugundji-Ugi model (Leber et al., 2009) and it calculates changes in stereochemistry following recent developments in the Chemistry Development Kit (CDK) (Steinbeck et al., 2003).

A strategy to measure enzyme similarity based on mechanistic data obtained from MACiE has also been presented (O’Boyle et al., 2007). The procedure builds upon a measure of similarity between mechanistic steps based on bond changes and features of the Ingold classification of reaction mechanisms. The similarity score is derived from an alignment algorithm similar to the one used in classical methods to compare protein sequences. This strategy helped to identify cases of evolutionary convergence and divergence of catalytic mechanisms (Almonacid & Babbitt, 2011; Almonacid et al., 2010), it has been recently employed to predict enzyme function from mechanism (Nath & Mitchell, 2012).

### 1.4 Isomerases

Scientists were already interested in isomerases back in 1970s when the three-dimensional structure of chicken triosephosphate isomerase (TIM) (EC 5.3.1.1) was first published (Banner et al., 1975). This enzyme catalyses the interconversion of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde 3-phosphate (GAP), one of the three isomerisations among the total of ten biochemical reactions present in the glycolysis pathway (Bar-Even et al., 2012). In the 1990s, mandelate racemase (EC 5.1.2.2) and muconate-lactonizing enzyme (EC 5.5.1.1), members of the enolase superfamily, were among the first enzymes reported to be highly structurally similar yet catalysing different overall
reactions (Petsko et al., 1993). Several isomerases belonging to this superfamily have been studied over the last two decades (Gerlt et al., 2012). More recently, further structural and mechanistic studies on TIM (Aguirre et al., 2014; Amyes & Richard, 2013) and two other isomerases, ketosteroid isomerase (EC 5.3.3.1) (Herschlag & Natarajan, 2013) and chorismate mutase (EC 5.4.99.5) (Kiss et al., 2013; Silverman, 2002; Vamvaca et al., 2004), have also been fundamental for understanding basic principles of enzyme catalysis. Here we review some biological relevance and applications of isomerases.

Figure 1.1: Metabolic pathways of the central metabolism (map01100), as in the 10th December 2012 version of KEGG database (Kanehisa et al., 2012). A total of 146 pathways comprise 1590 compounds represented as nodes and 2255 reactions as edges. Different colours represent different metabolic pathways and black edges indicate isomerase reactions. Image generated using Interactive Pathways Explorer v2 (iPATH2) (Yamada et al., 2011).

1.4.1 Metabolism

Isomerases are important biological components of the metabolism and genome of most living organisms. Most enzymes perform their catalytic function in the context of a metabolic pathway. Isomerases catalyse up to 4% of the biochemical reactions present in the central metabolism of most living organisms. In particular, isomerases are prevalent in carbohydrate metabolism and metabolism of terpenoids and polyketides (Figure 1.1). According to the total of 281 isomerase reactions present in the 10th December 2012
version of KEGG database (Kanehisa et al., 2012), 95 (33.8%) belong to the central metabolism whereas the remaining 186 belong to other metabolic pathways that are specific to certain groups of species, such as the biosynthesis of secondary metabolites, especially in plants.

1.4.2 Genome

Genetic information is encoded as DNA sequences in the genome of living organisms. The regions of the genome that encode any kind of biological function are known as genes. These are classified depending on whether they are transcribed and/or translated, for instance, we may refer to non-transcribed regulatory genes, transcribed RNA-genes and translated protein-coding genes (Patthy, 1999). Since isomerases are enzymes and enzymes are proteins, genes with isomerase function are encoded in the genome as protein-coding genes, which then translate into proteins with isomerase function.

UniprotKB contains functional annotations of protein-coding genes, which are critically reviewed by a dedicated team of curators (The Uniprot Consortium, 2013). This resource defines “complete proteome” as the set of proteins that are thought to be expressed in fully-sequenced organisms. For a gold-standard set of five organisms, each reviewed entry corresponds to a canonical isoform of a known protein-coding gene, in addition unreviewed entries represent other isoforms of the same genes, which arise from biological processes such as alternative splicing (Light & Elofsson, 2013). These organisms are Homo sapiens, Saccharomyces cerevisiae (baker’s yeast), Schizosaccharomyces pombe, Escherichia coli and Bacillus subtilis. For example, as in the 10th December 2012 version of UniprotKB, the complete proteome of Homo sapiens contains 20226 reviewed proteins and 47853 unreviewed proteins. UniprotKB assures that these many reviewed entries correspond to 20226 currently known human protein-coding genes whereas unreviewed entries equate to 47853 protein isoforms that are alternative products of these 20226 protein-coding genes.

In general, eukaryotes have more proteins than prokaryotes (Figure 1.2a). The relative proportion of enzymes encoding for isomerase activity depends on the species. Whereas 2.6% of the genes encoding for enzymatic activity correspond to isomerases in Homo sapiens, this proportion is higher in bacterial genomes such as Escherichia coli where they account for 6.2% (Figure 1.2b). These figures correlate well with the relative proportion of protein-coding genes encoding for enzymatic activity. Whereas in human, 20% of genes correspond to enzymes, this value increases to 37% in bacteria.
Figure 1.2: Distribution of isomerases in the genome. (a) Barplot representing the number of protein-coding genes (yellow), those annotated with enzymatic function (red) and particularly with isomerase function (blue). (b) Pie charts illustrating the relative amount of EC classes for each genome.

1.4.3 EC classification

The NC-IUBMB classifies isomerases as one of the six classes of enzymes belonging to the EC classification (McDonald & Tipton, 2014). They catalyse isomerisations, also known as geometrical and topological interconversions between isomers (Silverman, 2002). The International Union of Pure and Applied Chemistry (IUPAC) defines isomers as chemical species that share the same atomic composition but differ in their structural arrangement of atoms (IUPAC, 2014). As in the 1st November 2012, isomerases are identified as EC 5 class and classified according to three hierarchical levels: 6 subclasses (EC 5.b), 17 sub-subclasses (EC 5.b.c) and 245 EC numbers (EC 5.b.c.d) (Figure 1.3).

- **EC 5.1** consists of *racemases* and *epimerases*, which catalyse hydrogen shifts in molecules with one or more stereocentres, respectively.

- **EC 5.2** consists of *cis-trans isomerases* or enzymes catalysing geometry rearrangements in double bonds.

- **EC 5.3** consists of *intramolecular oxidoreductases*, which catalyse oxidation/reduction
reactions between isomers.

- **EC 5.4** consists of intramolecular transferases or enzymes transferring acyl-, phospho-, amino-, hydroxy- or other groups within the substrate.

- **EC 5.5** consists of intramolecular lyases, which catalyse intramolecular eliminations between isomers.

- **EC 5.99** consists of other isomerases or enzymes catalysing different reactions to the rest of subclasses.

The distribution of isomerases into 6 subclasses depends upon two distinct criteria: type of isomerisation and overall chemistry of the reaction. Although subclasses EC 5.1 and 5.2 are defined according to the former criteria, subclasses EC 5.3, 5.4 and 5.5 are based on the latter. Lastly, EC 5.99 contains isomerases that do not fit any of the above. Three of the six isomerase EC subclasses are similar to three EC primary classes (intramolecular oxidoreductases - EC 5.3 are designated from oxidoreductases - EC 1; intramolecular transferases - EC 5.4 from transferases - EC 2; and intramolecular lyases - EC 5.5 from lyases - EC 4, but refer to intramolecular reactions). Only three subclasses are further divided in sub-subclasses depending on the chemical nature of the substrate and chemical groups involved in the reaction (Figure 1.3). While EC 5.1 was divided according to the type of substrate, EC 5.3 was split depending on the bond change and reaction centre and EC 5.4 according to the transferred chemical group.

Isomerase sub-subclasses are divided into 245 EC numbers based on the chemical nature of the substrate. For example, EC 5.1.1 comprises 18 EC numbers corresponding to enzymes that catalyse racemisations of different amino acids and derivatives. The isomerase EC numbers are associated with about 300 biochemical reactions - for example EC 5.1.1.9 describes the racemisation of arginine, lysine or ornithine and it is therefore linked to three distinct reactions. Consequently, the relationship between EC number and biochemical reaction is not one-to-one so EC numbers are associated to more than one reaction and vice versa (see Chapter 5. Characterising Complex Biochemical Reaction Data).

Isomerases were also selected as a case study due to several practical reasons. There has been already substantial research focused on other EC classes like oxidoreductases (EC 1) and hydrolases (EC 3) (Hu et al., 2010; Mu et al., 2006; Sacher et al., 2009) and at the start of this study, other members of the Thornton group began a parallel study on ligases (EC 6), which was recently published (Holliday et al., 2014). The total number of isomerase EC numbers is small compared to other EC classes, which makes them attractive for manual analysis. Most of the isomerase reactions are unimolecular (one substrate and
Figure 1.3: Tree representing the EC classification of isomerases. Barplots illustrate the total amount of EC numbers (red) and KEGG reactions (green) at the subclass and sub-subclass levels. An isomerase reaction is given as an example for each sub-subclass. Substructures undergoing the reaction are highlighted in purple ovals. For instance, alanine racemase (EC 5.1.1.1) interconverts L-alanine and D-alanine.
one product) and therefore relatively easy to compare. Also, the automatic detection of stereochemistry changes (chiral inversions and cis/trans isomerisations) in biochemical reactions has been a long-standing problem in chemoinformatics due to technical challenges (Apostolakis et al., 2008) and problems with the stereochemical validity of metabolites in widely-used resources (Ott & Vriend, 2006). Since changes of stereochemistry are relevant in isomerase reactions and driven to overcome the existing technical limitations, isomerases were used as a test dataset in the development of EC-BLAST (Rahman et al., 2014), where modules for the automatic detection of stereochemistry changes were implemented.

General strategies to assign isomerase specificity have also been recently presented (Bouvier et al., 2014; Lukk et al., 2012; Song et al., 2007; Zhao et al., 2013), as well as comparative genomic techniques to discover new isomerases in bacterial genomes (Rodionova et al., 2012). Other investigations have partially explored isomerases in several superfamilies such as the haloacid dehalogenase, crotonase, vicinal oxygen chelate, amido- hydrodrolase, alkaline phosphatase, cupin, short-chain dehydrogenase/reductase and PLP-dependent aspartate amino-transferase superfamilies (Galperin & Koonin, 2012; Glasner et al., 2006b; Nguyen et al., 2009; Singh et al., 2012; Uberto & Moomaw, 2013).

1.4.4 Applications

Metabolic engineering

Metabolic engineering is the technology for the manipulation of organisms to synthesize high-value compounds of both natural and heterologous origin. Several research efforts engineered yeast and bacterial organisms for the synthesis of biofuels, an alternative to petroleum-based fuels, from cheap resources like lignocellulose, algal biomass, feedstocks and greenhouse gases (Kim et al., 2012; Peralta-Yahya et al., 2012). Seventy percent of lignocellulose’s composition is made of sugars, particularly glucose, xylose and arabinose. Bacterial xylose isomerase (EC 5.3.1.5), which converts D-xylose into D-xylulose, was engineered using directed evolution to increase the yield of alcohol-based biofuels in yeast (Lee et al., 2012; Young et al., 2010). The additional ability of xylose isomerase to transform D-glucose into D-fructose was also used in the industrial production of high fructose corn syrup, a common food sweetener (Hilterhaus & Liese, 2012).

Another isomerase that has been further explored is the bacterial isopentenyl-diphosphate Δ-isomerase (IPPS, EC 5.3.3.2), which is present in the terpenoid biosynthesis pathway and catalyses the conversion of isopentenyl diphosphate (IPP) into dimethylallyl diphosphate (DMAPP). IPP and DMAPP are key precursors for the synthesis of terpenoids which are widely-used as cosmetics, pharmaceuticals such as levopimaradiene (Leonard
et al., 2010) and isoprenoid-based biofuels like isopentanol, farnesane, bisabolane and pinene (Peralta-Yahya et al., 2012).

Organic synthesis

Asano and Hölsch thoroughly reviewed the multiple applications of isomerases to organic synthesis (Asano & Hölsch, 2012). A few examples are highlighted here. Enzymatic racemisation is considered as an alternative to existing methods of chemical racemisation, which tend to generate many undesired side products. Several racemases and epimerases (EC 5.1) are used to resolve racemic mixtures in mild chemical conditions and to synthesise stereochemically pure amino acids. For example, glutamate racemase (EC 5.1.1.3) was adopted for the large-scale production of D-glutamate, D-phenylalanine and D-tyrosine, where D-glutamate is an intermediate for the preparation of important pharmaceuticals such as penicillin derivatives (Schnell et al., 2003). Amino acid racemase (EC 5.1.1.10) was used to synthesise L-tryptophan.

In an attempt to show how catalytic promiscuity can be applied to asymmetric catalysis, Vongvilai and coworkers coupled the native acylation activity of a lipase enzyme with its promiscuous racemase activity in order to develop a method for the synthesis of N-methyl α-aminonitriles, natural precursors of α-amino acids (Vongvilai et al., 2011).

Enzyme design

Advances on enzyme design have been fundamental for the application of enzymes in organic chemistry, protein thermostability and the interconversion of catalytic activities (Bornscheuer et al., 2012; Kiss et al., 2013). At the root of this discipline lies the development of experimental techniques of directed evolution such as random mutagenesis and rational design of mutational libraries. These approaches broadly consist of introducing mutations in the enzyme followed by a selection strategy based on the activity of interest. Most engineered enzymes are hydrolases, however isomerases and ligases are under-represented (Kaltenbach & Tokuriki, 2014). Over the past few years, several attempts have anyway managed to successfully interconvert the activity of some isomerases. For example, triosephosphate isomerase (EC 5.3.1.1) and phosphoribosylanthranilate isomerase (EC 5.3.1.24) have a (βα)₈-barrel fold, which was shown to be useful starting scaffold for enzyme design (Höcker et al., 2001). More recently, researchers transformed racemases and epimerases acting on amino acids and derivatives (EC 5.1.1) into enzymes with lyase activity (EC 4) (Seebeck & Hilvert, 2003; Vick & Gerlt, 2007). In addition, Glasner and coworkers presented structural evidence of how evolution turned a lyase, namely α-succinylbenzoate synthases (EC 4.2.1.113), into an isomerase, N-succinylamino acid racemase (EC 5.1.1.-) in the enolase superfamily (Glasner et al., 2006a).
Isomerases in diseases and drug discovery

Ultimately, some racemases and epimerases acting on amino acids and derivatives (EC 5.1.1) are also targets for the development of antimicrobial drugs and the treatment of neuropathological disorders (Conti et al., 2011). For instance, glutamate racemase (EC 5.1.1.3) plays an essential role in the biosynthesis of peptidoglycan, a fundamental component of the bacterial cell wall. Therefore this racemase has been considered as a target for the development of antibacterial drugs (Lundqvist et al., 2007).

1.5 Structure of the thesis

The following chapters present an approach to understand the link between the chemistry and evolution of isomerases and a focused study aiming to dissect the diversity in the relationship between EC number and biochemical reaction.

Chapter 2 is a description of the data resources and methods used in this thesis. It first covers how biochemical reaction data and enzyme information were collected and curated. It also describes the technical tests performed on the EC-BLAST tool to compare enzyme reactions during its development (Rahman et al., 2014). Next, it presents a strategy to explore the evolution of isomerases using FunTree (Furnham et al., 2012b) and other enzyme resources. The various statistical methods used to analyse data are discussed in the order they appear in later chapters.

Chapters 3 and 4 focus on the chemistry and evolution of isomerase function, respectively. The first presents a broad overview of the overall chemistry of isomerases followed by a discussion about existing challenges in the EC classification of isomerases. The latter explores how isomerase function evolves in enzyme superfamilies.

The relationship between EC number and biochemical reaction is complex. Chapter 5 explores the principles and challenges of this relationship and proposes recommendations for the useful description of intricate EC numbers.

Finally, chapter 6 summarises and critiques the main results and presents some ideas about future research.

Some of the contents of chapters 1, 2 and 4 have been published in Martinez Cuesta et al. (2014) and Rahman et al. (2014).
Chapter 2

Data Resources and Methods

2.1 Introduction

In this chapter, the approach used to collect and curate information about the molecular function and evolution of isomerases is presented. Also, the various methods and statistical techniques employed to analyse the data are introduced.

Publications in the scientific literature and textbooks are the most reliable means by which biochemists, enzymologists and molecular biologists have traditionally shared enzyme knowledge. In recent years, the need to make this vast wealth of information publicly available and easily accessible to the scientific community and society led to the development of resources and databases that capture data such as biochemical assays, kinetics and enzyme sequences. Even more recently, with the advent of standards for the reporting of enzyme data (Apweiler et al., 2010; Tipton et al., 2014), many journals now demand electronic submission of enzyme data as a routine publication practice, which facilitates integration. Alternatively, curators from resources like UniprotKB (The Uniprot Consortium, 2013) and KEGG (Kanehisa et al., 2012) manually scrutinise the literature in order to extract the information that populates databases. From the perspective of a user aiming to perform a medium to large-scale analysis on a group of enzymes of interest, the starting point is usually the development of a workflow to retrieve and filter data in a semi-automatic manner. A technical description follows concerning the extraction and curation of isomerase data.

2.2 Biochemical reactions

2.2.1 Resources and dataset

The molecular function of enzymes corresponds to their ability to catalyse biochemical reactions as identified by the EC number (Tipton & Boyce, 2000) (see 1.1.2 Enzyme classi-
Biochemical reactions are available from databases such as KEGG, BRENDA and MetaCyc (Caspi et al., 2014; Kanehisa et al., 2012; Schomburg et al., 2013a). In April 2014, the NC-IUBMB listed 5385 active four-digit EC numbers in the classification. Structural information about the substrates and products of the reactions was accessed on the 9th April 2014 from the 70.0+ release of KEGG using the Advanced Programming Interface (API) (Kawashima et al., 2003). The data includes 6494 unique biochemical reactions that are linked to 4237 EC numbers, comprising almost 80% of all EC numbers (Figure 2.1).

As a way to summarise the diversity existing in a multi-reaction EC number, biological databases such as KEGG rely on the so-called “IUBMB reaction”. This is the reaction assigned to the EC number by the NC-IUBMB in the first place, which is chosen by KEGG as the representative reaction for the group of reactions associated with the same EC number (Figure 5.7). Whereas this assignment is useful when selecting an example reaction from an EC number and it was adopted as a principle in the development of other reaction databases such as Rhea (Alcántara et al., 2012), it is sometimes missing or conflicting and it also overlooks the existing diversity present in the rest of reactions. Similarly, some EC numbers are not associated to any IUBMB reaction and also, EC numbers are sometimes linked to the same IUBMB reaction, 2,3-diphosphoglycerate-dependent and independent phosphoglycerate mutases (EC 5.4.2.11 and EC 5.4.2.12) are both assigned the same IUBMB reaction comprising the isomerisation of 2-phospho-D-glycerate to 3-phospho-D-glycerate. Taken together, a more robust and consistent approach to describe multi-reaction EC numbers is necessary (see Chapter 5. Characterising Complex Biochemical Reaction Data). In order to facilitate the interpretation of results, our analysis of the chemistry and evolution of isomerase function (Chapters 3 and 4, respectively) assumes a one-to-one relationship whereby a single representative reaction uniquely designates any given isomerase EC number.

The structures of substrates and products were downloaded in MDL MOL format, these are connection tables listing atoms, their 2D coordinates and bonds in a tabular format (Warr, 2014). Explicit hydrogens were manipulated using the Molecule File Converter of Marvin version 5.9.4 (http://www.chemaxon.com) and reaction files were built and stored in Rxnfile format. Among other reaction formats available such as RDfile and RInChI (Grethe et al., 2013), Rxnfile was chosen as it is the preferred input format for EC-BLAST. The Rxnfile format is an extension of the MDL MOL format. At the top of the file, a header indicates the number of substrates and products of the reaction followed by their structures.
Figure 2.1: A flow diagram illustrating the collection of isomerase EC numbers (EC 5) and associated biochemical reactions from NC-IUBMB and KEGG.
2.2.2 Methods and tools

One of the multiple approaches to obtain functional similarity between enzymes is to compare their catalysed biochemical reactions. As presented in Chapter 1. Introduction, comparisons can be performed according to different criteria. EC-BLAST introduces three measures of functional similarity based on chemical attributes such as bond changes, reaction centres and the structures of substrates and products derived from the reaction (Rahman et al., 2014) (Figure 2.2). Chemical structures were also analysed using RDKit, a widely-used chemoinformatics software (Landrum, 2013) and visualised using Marvin version 5.9.4 (http://www.chemaxon.com).

At the core of the EC-BLAST tool, four different algorithms are built upon the concept of maximum common substructure (MCS) to perform atom-atom mapping (Chen et al., 2013; Rahman et al., 2009). Bond changes and reaction centres are derived automatically using the Dugundji-Ugi model. The best solution is chosen on the basis of the number of bond changes and fragments generated and bond energies according to the principle of minimum chemical distance (Jochum et al., 1980). In this model, substrates and products are represented using bond-electron matrices where diagonal elements correspond to the number of free valence electrons and off-diagonal entries give the bond orders between atoms. The reaction matrix is obtained when the substrates matrix is subtracted from the products matrix. A positive element on this matrix indicates bond formation, whereas a negative element corresponds to bond cleavage. Changes in the stereochemistry of atoms and bonds, also known as stereochanges, are coded in parallel.

In order to allow comparisons between the chemistry of enzymes, EC-BLAST encodes reactions using three distinct fingerprints based on bond changes, reaction centres and structures of substrates and products, respectively. Bond changes refer to the cleavage and formation of chemical bonds, changes in bond order and stereochanges, which are due to chemical processes such as chiral inversions or cis-trans isomerisations. Cleaved and formed bonds are indicated as lines connecting atoms, for instance C–C means a single carbon-carbon bond that is cleaved or formed in the reaction. Bond order changes are represented as double arrows connecting bonds, for example C–C ↔ C=C means a single carbon-carbon bond turning into double carbon-carbon bond or vice versa. Stereochanges are represented as atoms that change their absolute configuration, for instance C(R/S) means a carbon atom that changes from R to S configuration.

A reaction centre is the collection of atoms and bonds that are changed during the reaction (Warr, 2014), also known as the local atomic environment around the atoms involved in bond changes. EC-BLAST calculates circular fingerprints around the non-hydrogen
Figure 2.2: Flow diagram illustrating how EC-BLAST works. Alanine racemase (EC 5.1.1.1) is used as a query example.
atoms present in bond changes at three different levels (0, 1 and 2) (Rahman et al., 2014). Reaction centres at level 0 simply comprise the atoms involved in bond changes, level 1 extends by one covalent bond from level 0. Finally, level 2 extends by two covalent bonds from level 0 (Figure 2.3).

In order to measure the specificity of bond changes or reaction centres in each EC subclass or sub-subclass, the Shannon-Wiener (SW) information statistic was used (Roberts, 2012). This is an index which is commonly used to measure species diversity in ecology. However, for this purpose it allows to quantify the uncertainty associated to predict the subclass from a bond change or reaction centre. It is calculated as

$$\text{SW} = -\sum_{i=1}^{6} p_i \ln p_i$$

where $p_i$ is the proportion of bond changes or reaction centres belonging to the $i$th subclass summed up across all 6 subclasses. Then it is scaled in the interval 0 to 1. In general, rare bond changes or reaction centres tend to be only present in one subclass and have a SW statistic equal to 1. On the other hand, common bond changes, such as C(R/S) are distributed across more than one subclass so they have a SW statistic considerably lower than 1 (see Chapter 3. The Chemistry of Isomerases).

EC-BLAST calculates similarity between reaction fingerprints using a Tanimoto score ranging between 0 (no similarity) and 1 (identical reactions) (Rahman et al., 2014). The results obtained with different chemical attributes were stored in similarity matrices, which were then compared using two strategies. First, two-sample Kolmogorov-Smirnov (KS) tests were used to explore whether similarity distributions are significantly different from one another. Specifically, KS tests evaluate the null hypothesis that similarity distributions are drawn from the same continuous distribution (Crawley, 2007). Second, Mantel tests were adopted to obtain correlations between similarity matrices using the Pearson’s product-moment correlation as the method. Significance was assessed by permuting rows and columns of one of the similarity matrices under comparison (Legendre & Legendre, 2012; Oksanen, 2011; R Core Team, 2012).

To find groups of similar reactions according to the chemical attributes mentioned above, hierarchical clustering was performed using the R environment for statistical computing (R Core Team, 2012). Three approaches were employed to select the best clustering algorithm and to choose the optimal number of clusters. First, using external evaluation, clustering algorithms were compared on their ability to obtain greatest purity in EC subclasses or sub-subclasses using the F-measure as implemented in in-house scripts provided by Dr. Syed Asad Rahman. This is the harmonic mean of precision ($p$) and recall ($r$) defined as $F - measure = \frac{2pr}{p+r}$. In the context of EC subclasses, precision is the fraction of isomerase reactions classified in the correct subclass ($p = \frac{TP}{TP+FP}$) and captures the subclass purity of clusters. Recall refers to the fraction of isomerase reactions
Figure 2.3: The reaction catalysed by isopentenyl-diphosphate Δ-isomerase (EC 5.3.3.2) has 6 carbon atoms involved in bond changes (3 in the substrate and 3 in the product). (a) Bond changes as calculated by EC-BLAST (b) Reaction centres obtained based on the atoms involved in bond changes.
belonging to the same subclass grouped in the same cluster \( r = \frac{TP}{TP + FN} \) and represents how spread subclasses are across different clusters. TP (true positives) are the number of pairs of reactions in the same EC subclass and the same cluster, FP (false positives) are the number of pairs in different EC subclass but located in the same cluster, finally FN (false negatives) are the number of pairs in the same EC subclass but different clusters.

Second, as internal evaluation, hierarchical trees were pruned at the height that simultaneously minimises the number of clusters and the spread within each cluster as discussed in Kelley et al. (1996) and Fischer et al. (2010), and implemented in White & Gramacy (2012). Alternatively, Rand Index and Silhouette width (Mavridis et al., 2013) were also investigated as external and internal evaluation metrics respectively, however they did not perform better than the F-measure and Kelley-Gardner-Sutcliffe penalty function. Third, the best correspondence between clusters and EC classification was explored using the mclust package (Fraley et al., 2012), which helped to determine the extent to which subclasses and sub-subclasses are prevalent in clusters.

The hierarchical clustering solutions computed for each chemical attribute were contrasted using three methods. First, cross tabulations of EC numbers clustered according to two chemical attributes, for example bond changes versus reaction centres, are captured in contingency tables, also known as matching matrices. Second, topological distances between clustering trees were calculated, which are defined as twice the number of internal branches representing different bipartitions of the tips (Paradis, 2012; Paradis et al., 2004; Penny & Hendy, 1985) are also useful. Third, tanglegrams involve drawing two clustering trees opposite to each other, which help to visualise comparisons between different clustering solutions (Scornavacca et al., 2011). Clustering evaluation metrics such as F-measure and Rand Index were also explored here.

Other techniques to explore and visualise the similarity and clustering of biochemical reactions were investigated. For instance, dimensionality reduction techniques such as principal component analysis and multi-dimensional scaling (Everitt & Hothorn, 2011), correspondence analysis (Greenacre, 2007; Husson et al., 2011) and self-organising maps (Chen & Gasteiger, 1997; Wehrens & Buydens, 2007). The overall interpretation of results using these alternative approaches was similar to the analysis using hierarchical clustering, however the visualisation was not as intuitive, therefore they were not shown in this dissertation.

2.2.3 Testing EC-BLAST with isomerase reactions

During the period 2012-2014, isomerase reactions were used as a test set in the development of EC-BLAST (Rahman et al., 2014). At the start of the test in April 2012,
the first challenge involved a critical assessment of the chemical validity of reaction data contained in an internal EC-BLAST database built upon the release 58.1 of KEGG (accessed on 1st June 2011). Although approaches to perform automatic checks of reaction data quality had already been presented (Ott & Vriend, 2006), a manual workflow aiming to report reaction data inconsistencies was designed for our specific purposes. The second challenge was to test EC-BLAST’s ability to extract chemical attributes such as bond changes from biochemical reactions. Many isomerase reactions involve changes of stereochemistry caused by chemical processes such as chiral inversion and cis-trans isomerisation. To certain extent, this test helped Dr. Syed Asad Rahman to implement modules dealing with the automatic detection of stereochemistry changes in EC-BLAST.

The reference list of isomerase EC numbers used in this test was obtained from the 21st March 2012 release of ENZYME database (Bairoch, 2000). This resource actively follows the recommendations of the NC-IUBMB concerning standard nomenclature and classification of enzymes. The test set comprised 202 four-digit isomerase EC numbers linked to 289 unique biochemical reactions, which were obtained from release 62.0 of KEGG (accessed using KEGG API). The manual curation of isomerase reactions comprised three major tasks: detection of KEGG errors, outdated data and software errors. KEGG errors and outdated data were easy to discover because they originate from data quality problems or mismatch between the NC-IUBMB, KEGG and internal EC-BLAST databases. However, discovery of software errors required a more thorough process of manual inspection and validation of isomerase reactions. The types of errors encountered in the initial stages of the curation process are illustrated in Figure 2.4 and Table 2.1.

The test covered eight rounds of manual checks and seven rounds of code optimisations, which ultimately led to a significant decrease in the number of software errors. The first manual check resulted in a total of 312 errors. A third of them pointed to inconsistencies with the atom-atom mapping performed by EC-BLAST as indicated by their bond changes. Another third consisted of outdated data issues and the remaining third comprised KEGG errors and other software errors. This test took almost a year to complete and by the eighth manual check, the total number of errors considerably reduced to 200 (Figure 2.6). Although only three stereochemistry errors were resolved during this set of optimisations (pink, black and gray bars), this situation improved as the R/S perception library was optimised and modules to detect E/Z stereochemistry were implemented in later stages of software development (Rahman et al., 2014).

After the eighth round of manual checks, most software errors were resolved (Table 2.2). KEGG errors were fixed in parallel to this test by manually curating reactions from release 58.1 of KEGG, storing them in a database of reactions within EC-BLAST and ac-
Figure 2.4: Flow diagram representing the manual process of error-checking in EC-BLAST using isomerase reactions.
Table 2.1: Summary of errors detected at the beginning of the manual curation of isomerase reactions using EC-BLAST.

<table>
<thead>
<tr>
<th>Type of error</th>
<th>Error name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>KEGG errors</td>
<td>KEgg errors</td>
<td>KEgg does not link the EC number to reaction data. Example: protein disulfide-isomerase (EC 5.3.4.1) catalyses the rearrangement of S–S bonds in proteins.</td>
</tr>
<tr>
<td></td>
<td>No structures</td>
<td>KEgg does not provide structural information for all substrates and products of a reaction. Example: cellobiose epimerase (EC 5.1.3.11).</td>
</tr>
<tr>
<td></td>
<td>Undefined stereochemistry</td>
<td>The stereochemistry of some chiral atoms in KEgg is unknown (depicted as flat bond) or any (wiggly bond). Defined stereochemistry is represented as wedge or hatch bonds, also known as up or down, respectively (Brecher, 2008) (<a href="http://www.chemaxon.com">http://www.chemaxon.com</a>) (Figure 2.5a).</td>
</tr>
<tr>
<td>Outdated data errors</td>
<td>EC is not in EC-BLAST database</td>
<td>Example: 2,3-diacetamido-2,3-dideoxy-α-D-glucuronate 2-epimerase (EC 5.1.3.23).</td>
</tr>
<tr>
<td></td>
<td>KEGG reaction is not in EC-BLAST database</td>
<td>Example: R09600 involves the conversion of UDP-α-D-GlcNAc3NAcA into UDP-α-D-ManNAc3NAcA.</td>
</tr>
<tr>
<td>Software errors</td>
<td>Error in BC table</td>
<td>Bond changes extracted from EC-BLAST do not match bond changes derived manually due to problems in the atom-atom mapping. Example: styrene-oxide isomerase (EC 5.3.99.7) (Figure 2.5b).</td>
</tr>
<tr>
<td></td>
<td>N rings</td>
<td>EC-BLAST incorrectly cleaves all N-H bonds. Double bonds in five and six member heterocycles are represented incorrectly. Example: L-dopachrome isomerase (EC 5.3.3.12).</td>
</tr>
<tr>
<td></td>
<td>Stereochemistry</td>
<td>(a) Some cases of R/S stereochemistry are not detected. Example: amino acid racemase (EC 5.1.1.10) (Figure 2.5c). (b) Wrong R/S stereochemistry is detected. Example: proline racemase (EC 5.1.1.4) (Figure 2.5d). (c) E/Z (also known as cis/trans) and omega stereochemistry is not detected. At this early stage of software development, EC-BLAST was not able to account for this type of stereochemistry. Example: maleate isomerase (EC 5.2.1.1) (Figure 2.5e).</td>
</tr>
</tbody>
</table>
Figure 2.5: Examples of errors discovered while testing EC-BLAST. (a) Undefined stereochemistry in 3-oxosteroid Δ5-Δ4-isomerase (EC 5.3.3.1). In red, an example of unknown stereochemistry in a reacting atom (see product). Although there are four cases of defined stereochemistry in non-reacting atoms shown as green wedge bonds in the substrate, stereochemistry is depicted as unknown in the product. (b) Although EC-BLAST reports the cleavage of a C–C bond in styrene-oxide isomerase (EC 5.3.99.7), according to mechanistic studies the substrate undergoes an epoxide ring opening via the cleavage of a C–O only (Hartmans et al., 1989) and C–C cleavage does not take place. (c) R/S stereochemistry is not detected in amino acid racemase (EC 5.1.1.10). (d) Wrong R/S stereochemistry is detected in proline racemase (EC 5.1.1.4). (e) E/Z stereochemistry is not detected in maleate isomerase (EC 5.2.1.1).
tively reporting KEGG about data inconsistencies found during this process. As a result, KEGG corrected many of the inaccurate entries in following releases of their database. Resolving outdated data issues first involved the exploration of other reaction databases such as BKM-React (Lang et al., 2011) and Rhea (Alcántara et al., 2012). Second, in April 2014 release 58.1 of KEGG was upgraded to release 70.0+ (Figure 2.1) so to represent a more recent version of the space of biochemical reactions in this dissertation.

Only the initial stages of the process of testing EC-BLAST have been described here, however manual validation of the chemical content of EC-BLAST and KEGG still continues today covering a broader spectrum of biochemical reactions from different EC classes. Also, when newer versions of the algorithm are developed, manual analysis against a challenging test set of reactions is undertaken. This evaluation set has continuously been updated during EC-BLAST development. There are several reactions though that remain challenging due to theoretical difficulties while performing the atom-atom mapping and extracting chemical attributes (Figure 2.7). These cases represent a motivation for further improvement of existing algorithms.
Table 2.2: Distribution of software errors and consequent actions of code optimisation during the EC-BLAST test.

<table>
<thead>
<tr>
<th>Round</th>
<th>Software error</th>
<th>Total</th>
<th>Code optimisation strategy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BC</td>
<td>N rings</td>
<td>Stereo</td>
</tr>
<tr>
<td>1</td>
<td>104</td>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>66</td>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>31</td>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>6</td>
<td>17</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>0</td>
<td>17</td>
</tr>
</tbody>
</table>

2.3 Evolution

2.3.1 Resources and dataset

Protein similarity networks have been used very successfully to map biological information to large sets of proteins (Brown & Babbitt, 2012; Uberto & Moomaw, 2013). However, it is also necessary to include associated changes of catalytic function during evolution preferably in an automated fashion. FunTree is a resource developed to accomplish that goal (Furnham et al., 2014; Holliday et al., 2012). By combining sequence, structure, phylogenetic, chemical and mechanistic information, it allows one to answer fundamental questions about the link between enzyme activities and their evolutionary history in the context of superfamilies (Figure 2.8a).

First, FunTree clusters the structures of the CATH domains involved in enzyme function as defined by MACiE and CSA, which are both resources that manually annotate enzyme mechanisms and catalytic sites, respectively (Furnham et al., 2014; Holliday et al., 2012).
Structural clusters are subsequently populated with sequence relatives from Gene3D (Lees et al., 2010) using BLASTp (Altschul et al., 1997) and structurally-informed multiple sequence alignments are then generated with FUGUE (Shi et al., 2001). Second, alignments are the starting point to create species-guided phylogenetic trees generated with the maximum likelihood (ML) method and the WAG substitution model using PHYML as implemented in TreeBest (Heng, 2006; Ruan et al., 2008). For tree visualisation purposes, sequences were filtered by taxonomic lineage while maintaining functional diversity, sequences with known structure and multidomain architecture diversity. Last, functional annotations are retrieved from the reviewed section of UniprotKB, PDBSum and KEGG (de Beer et al., 2014; Kanehisa et al., 2012; The Uniprot Consortium, 2013).

2.3.2 Methods and tools

FunTree uses phylogenetic methods to infer ancestral enzymes in superfamilies and estimate their most likely functions (Paradis, 2012). By systematically traversing the phylogenetic tree from ancestor to modern enzymes in a superfamily, explicit changes of function are identified between groups of enzymes. Ultimately, each functional change is represented by two sets of enzymes catalysing two distinct functions (Figure 2.8b). Pairs of functions and their corresponding sets of enzymes are comparatively analysed using functional and all-against-all sequence similarity. Functional similarity was obtained using EC-BLAST (see 2.2 Biochemical reactions) and results were interpreted in the light of mechanistic data extracted from MACiE and extensive literature searches, which com-
Clustering structural domains and gathering sequence data

Structurally-informed multiple sequence alignments

Phylogenetic analysis

Functional annotation

Phosphatidylinositol phosphodiesterase (PIP) superfamily

Figure 2.8: (a) Illustration of the FunTree pipeline (b) Example of the estimation of a functional change during the evolution of PIP superfamily. Phospholipase D activity (EC 3.1.4.4, cyan) was inferred to have evolved from sphingomyelin phosphodiesterase D activity (EC 3.1.4.41, orange). Image courtesy of Dr. Nicholas Furnham.
prehensively informed the analyses. Similarity between homologous enzyme sequences was calculated as sequence identity derived from the multiple sequence alignments used to generate the phylogenetic trees.
Chapter 3

The Chemistry of Isomerases

This chapter describes an analysis of isomerase reactions on the basis of three chemical attributes: bond changes, reaction centres and structures of reactants (substrates and products). Next, the quality of the EC classification of isomerases is discussed as well as ways to improve it. Last, three isomerase EC subclasses are further explored: racemases and epimerases (EC 5.1), intramolecular oxidoreductases (EC 5.3) and intramolecular transferases (EC 5.4). The discussion section addresses the ability of the EC classification to represent isomerase function as measured by the overall reaction.

3.1 Analysis of bond changes

3.1.1 Distribution

The six isomerase subclasses have different numbers of representative reactions and bond changes associated with them (Figure 3.1). EC 5.4 (intramolecular transferases) is the most abundant subclass with 71 reactions, closely followed by EC 5.1 (racemases and epimerases) and EC 5.3 (intramolecular oxidoreductases) with 58 and 57 reactions, respectively. In terms of bond changes EC 5.4, followed by EC 5.3 and EC 5.5 (intramolecular lyases) are the subclasses with largest number of bond changes. The overall distribution of the number of reactions with a given number of bond changes indicates diversity across different subclasses (Figure 3.2a). The average number of bond changes per reaction is 7.3. There are several extreme cases with more than 20 bond changes, which correspond to complex reactions belonging to sub-class EC 5.4.99. EC 5.1, 5.2 and 5.99 catalyse only a small number of bond changes (1 to 8). However EC 5.3, 5.4 and 5.5 correspond to reactions with more bond changes on average (Figure 3.2b).

A total of 30 different types of bond changes were found in our dataset of isomerase reactions. The most common bond changes are R/S stereochramge (C(R/S)), cleavage and formation of carbon-hydrogen bond (C–H) and cleavage and formation of oxygen-
Figure 3.1: Distribution of 219 representative isomerase reactions (1st column) and the 1603 occurrences of bond changes (2nd column) associated with them across EC subclasses. Bars are coloured according to subclass. This colouring scheme of the different subclasses is adopted throughout the thesis.

hydrogen bond (O–H) (Figure 3.3). Some types of bond changes occur more often in certain subclasses. Stereochange C(R/S) occurs in all the subclasses except EC 5.2 and 5.99 (Figure 3.3). It is also the only type of bond change observed in subclass EC 5.1 with the exception of one reaction: the conversion of L-phenylalanine into D-phenylalanine where L-phenylalanine racemase (EC 5.1.1.11) catalyses a single stereochange C(R/S) and unusually for a racemase, it also involves the cleavage and formation of two O–P bonds and two O–H bonds from ATP and water. This is the only isomerase reaction that is not unimolecular. Bond change C–H occurs in four of the six subclasses with the exception of EC 5.1 and EC 5.99, whereas bond change H–O occurs in all the subclasses except EC 5.2. From the point of view of the subclass, EC 5.1 and 5.2 are represented by a small subset of three bond changes: EC 5.1 is mainly C(R/S) whereas O–P and H–O are rare, EC 5.2 is C(E/Z), C–H and C–C ↔ C=C are rare. On the other hand, EC 5.3,
Figure 3.2: Distribution of the number of bond changes per isomerase reaction by EC subclass. (a) Bar plot showing the number of isomerase reactions against number of bond changes. (b) Box and whisker plot representing the distribution of the number of bond changes per isomerase reaction by EC subclass. The number of reactions belonging to each subclass is shown in brackets. Coloured boxes contain the interquartile range of each subclass. Whiskers represent 1.5 times the size of the coloured boxes. Single points beyond the whiskers represent outliers (Crawley, 2007).
5.4, 5.5 and 5.99 exhibit a much broader variety of bond changes. Overall, subclasses EC 5.1, 5.2 and 5.99 have less diversity of bond changes than the rest, which seem more complex.

Figure 3.3: Distribution of the number of isomerase reactions by bond change and EC subclass.

How much does each bond change contribute to a subclass? Do some bond changes discriminate subclasses more specifically? The relative frequency of occurrence of each bond change in subclasses was used to calculate the degree whereby bond changes are specific to certain subclasses using the Shannon-Wiener (SW) information statistic (Roberts, 2012) (see Chapter 2. Data Resources and Methods). In general, rare bond changes, e.g. C≡N ↔ C=N (Figure 3.3) are catalysed by enzymes from only one subclass and have a SW statistic equal to 1 (Table 3.1). On the other hand, abundant bond changes, such as C(R/S) are distributed across more than one subclass so they have a SW statistic lower than 1. Twelve bond changes (40% of all) are perfect discriminators of subclasses. For
instance, C≡N ↔ C=N is specific for the EC 5.99. However the relative frequency of specific bond changes is generally low - they are rare. O–O(ring) is specific for EC 5.3 and it is a chemical attribute of four different isomerase reactions catalysed by enzymes present in the arachidonic acid metabolism: prostaglandin synthases D, E and I (EC 5.3.99.2, 5.3.99.3 and 5.3.99.4) and thromboxane-A synthase (EC 5.3.99.5). These enzymes catalyse the opening of epidioxy bridges in prostaglandins.

Table 3.1: How specific are bond changes to isomerase subclasses?

<table>
<thead>
<tr>
<th>Bond change</th>
<th>SW</th>
<th>EC subclasses</th>
<th>Number of EC subclasses</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>O–P ↔ O=P</td>
<td>1.00</td>
<td>5.3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>C≡N ↔ C=N</td>
<td>1.00</td>
<td>5.99</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>N–O</td>
<td>1.00</td>
<td>5.4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>C=C</td>
<td>1.00</td>
<td>5.4</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>C–N(aromatic)</td>
<td>1.00</td>
<td>5.5</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>O–O(ring)</td>
<td>1.00</td>
<td>5.3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>O–O</td>
<td>1.00</td>
<td>5.4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>C–N(ring) ↔ C–N(ring)</td>
<td>1.00</td>
<td>5.4</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>C–P</td>
<td>1.00</td>
<td>5.4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C–S</td>
<td>1.00</td>
<td>5.99</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C–C(ring) ↔ C–C(aromatic)</td>
<td>1.00</td>
<td>5.3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>C–S ↔ C=S</td>
<td>1.00</td>
<td>5.99</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>H–N</td>
<td>0.69</td>
<td>5.4, 5.5</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>C–N</td>
<td>0.64</td>
<td>5.4, 5.99</td>
<td>2</td>
<td>31</td>
</tr>
<tr>
<td>C=O</td>
<td>0.62</td>
<td>5.4, 5.5</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>C–O(ring) ↔ C=O</td>
<td>0.61</td>
<td>5.3, 5.5</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>O–P</td>
<td>0.61</td>
<td>5.1, 5.4</td>
<td>2</td>
<td>29</td>
</tr>
<tr>
<td>C–C(ring) ↔ C=C</td>
<td>0.61</td>
<td>5.4, 5.5</td>
<td>2</td>
<td>117</td>
</tr>
<tr>
<td>C–C(ring) ↔ C–C</td>
<td>0.45</td>
<td>5.3, 5.4, 5.5</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>C–C(ring)</td>
<td>0.44</td>
<td>5.3, 5.4, 5.5</td>
<td>3</td>
<td>152</td>
</tr>
<tr>
<td>C–C(ring) ↔ C–C(ring)</td>
<td>0.44</td>
<td>5.3, 5.4, 5.5</td>
<td>3</td>
<td>31</td>
</tr>
<tr>
<td>C(E/Z)</td>
<td>0.40</td>
<td>5.2, 5.3, 5.4</td>
<td>3</td>
<td>34</td>
</tr>
<tr>
<td>C–C</td>
<td>0.40</td>
<td>5.3, 5.4, 5.5</td>
<td>3</td>
<td>158</td>
</tr>
<tr>
<td>C–O</td>
<td>0.39</td>
<td>5.3, 5.4, 5.5</td>
<td>3</td>
<td>26</td>
</tr>
<tr>
<td>C–H</td>
<td>0.28</td>
<td>5.2, 5.3, 5.4, 5.5</td>
<td>4</td>
<td>413</td>
</tr>
<tr>
<td>C–C ↔ C=C</td>
<td>0.25</td>
<td>5.2, 5.3, 5.4, 5.5</td>
<td>4</td>
<td>57</td>
</tr>
<tr>
<td>C(R/S)</td>
<td>0.24</td>
<td>5.1, 5.3, 5.4, 5.5</td>
<td>4</td>
<td>251</td>
</tr>
<tr>
<td>C–O ↔ C=O</td>
<td>0.23</td>
<td>5.3, 5.4, 5.5, 5.99</td>
<td>4</td>
<td>45</td>
</tr>
<tr>
<td>C–O(ring)</td>
<td>0.23</td>
<td>5.3, 5.4, 5.5, 5.99</td>
<td>4</td>
<td>81</td>
</tr>
<tr>
<td>H–O</td>
<td>0.11</td>
<td>5.1, 5.3, 5.4, 5.5, 5.99</td>
<td>5</td>
<td>119</td>
</tr>
</tbody>
</table>
3.1.2 Similarity and clustering

Given this distribution of bond changes the optimal subdivision of isomerase reactions into subclasses was explored using hierarchical clustering. A two step procedure was performed, first pairwise similarities between isomerase reactions were computed based on the frequency of bond changes using EC-BLAST (Figure 3.4a). The comparisons were stored in a similarity matrix. Secondly, a clustering algorithm was applied to the similarity matrix in order to group similar reactions (Figure 3.4b). After testing various clustering procedures by scanning how different cuts along the trees lead to greatest purity in EC subclasses, the Ward algorithm was selected to produce an optimal number of 6 chemically sensible clusters (see Chapter 2. Data Resources and Methods). Clustering results are illustrated in Figure 3.5 and summarised in Table 3.2 according to three main descriptors:

- The main subclass of a cluster was defined as the subclass with the highest number of reactions. For instance, EC 5.1 was considered the main EC subclass of cluster F.

- The main bond changes of a cluster were considered as those present in at least 80% of the reactions belonging to that cluster. For example, H–O and O–P were identified as main bond changes in cluster A.

- We also defined an outlier in a cluster as a reaction belonging to a different EC subclass than adjacent reactions in the clustering. Two main types of outliers were identified: first, outliers were defined as distinct if at least one of its bond changes is unique among the rest of bond changes from surrounding reactions. Conversely, an outlier was defined as misannotated if its bond changes are shared by at least two other neighboring reactions of a different subclass. As an example, in cluster D we identified EC 5.5.1.3 as a distinct reaction because among other bond changes, H–O is present and C–H is absent in reactions EC 5.3.2.5, 5.3.1.13 and 5.3.1.27. On the other hand, EC 5.2.1.5 in cluster C was considered as a misannotated reaction because it shares the presence of all its bond changes C(E/Z), C–C ↔ C=C and C–H with reactions EC 5.3.3.8, 5.3.3.13 and 5.3.3.14.

Table 3.2 explores the six clusters depicted in Figure 3.5. Only one cluster is pure in subclasses: F in EC 5.1. Three clusters are more than 75% pure: A in EC 5.4, B in EC 5.2 and D in EC 5.3. Finally, two clusters are mixed: C (mainly EC 5.3 and 5.4) and E (EC 5.4 and 5.5). From the subclass point of view, almost all racemase and epimerase reactions (EC 5.1) group in cluster F. The only exception is EC 5.1.1.11, which belongs to cluster A and it is defined as misannotated because of sharing identical bond changes to five intramolecular transferase reactions (EC 5.4). Cis-trans isomerase reactions (EC 5.2)
Figure 3.4: (a) Distribution of pairwise similarities of isomerase reactions by bond changes. (b) Bond change similarity matrix. Blue-to-red scale represents increasing similarity, identical reactions have a similarity of 1 (red). Reactions are annotated in colours according to their EC subclass (top row). Six clusters were identified (A to F) (see Figure 3.5).
Figure 3.5: Hierarchical clustering of isomerase reactions (rows) by bond changes (columns). The blue scale represents frequency of bond changes in reactions. Reactions are annotated in colours according to their EC subclass (left-hand column). Six clusters were identified (A to F) (see Figure 3.4b). Bond changes were ordered left-to-right according to increasing frequency (Figure 3.3). Outliers are annotated with an arrow (see main text).
Table 3.2: Analysis of the clusters obtained in Figure 3.5. The columns Cluster, Number of reactions and EC subclasses are self-explanatory. The main subclass is underlined and the number of reactions belonging to each subclass is shown in brackets. The columns Main bond changes and Outliers and comments are defined in the main text.

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Number of reactions</th>
<th>EC subclasses</th>
<th>Main bond changes</th>
<th>Outliers and comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>11</td>
<td>EC 5.1 (1) and 5.4 (10)</td>
<td>H–O and O–P</td>
<td>EC 5.1.1.11 is a <em>misannotated</em> reaction. Pure cluster</td>
</tr>
<tr>
<td>B</td>
<td>9</td>
<td>EC 5.2 (8) and 5.3 (1)</td>
<td>C(E/Z)</td>
<td>EC 5.3.3.7 is a <em>misannotated</em> reaction. Pure cluster</td>
</tr>
<tr>
<td>C</td>
<td>82</td>
<td>EC 5.2 (3), 5.3 (37), 5.4 (34), 5.5 (7) and 5.99 (1)</td>
<td>C–H</td>
<td>EC 5.2.1.5 is a <em>misannotated</em> reaction. Mixed cluster</td>
</tr>
<tr>
<td>D</td>
<td>22</td>
<td>EC 5.3 (18), 5.4 (1), 5.5 (2) and 5.99 (1)</td>
<td>C–H, O–H and C–O ↔ C=O</td>
<td>EC 5.5.1.3 is a <em>distinct</em> reaction. Almost pure cluster</td>
</tr>
<tr>
<td>E</td>
<td>38</td>
<td>EC 5.3 (1), 5.4 (26) and 5.5 (11)</td>
<td>C–H, C–C(ring), C–C and C–C(ring) ↔ C=C</td>
<td>EC 5.4.99.38 is a <em>distinct</em> reaction. Mixed cluster</td>
</tr>
<tr>
<td>F</td>
<td>57</td>
<td>EC 5.1 (57)</td>
<td>C(R/S)</td>
<td>No outliers. Pure cluster</td>
</tr>
</tbody>
</table>

are mostly grouped in cluster B. Exceptions are EC 5.2.1.5 (*misannotated*), 5.2.1.8 and 5.2.1.13 in cluster C. The rest of subclasses are mixed across several clusters, intramolecular oxidoreductases (EC 5.3) are present in four clusters: B, C, D and E; intramolecular transferases (EC 5.4) are in four clusters: A, C, D and E; intramolecular lyases (EC 5.5) are in three clusters: C, D and E; and other isomerases (EC 5.99) are in two clusters: C and D.

Out of the total of 219 representative isomerase reactions considered in this study, our bond change analysis questioned the EC subclass classification of 3 isomerase reactions:

- Located in cluster A, EC 5.1.1.11 corresponds to phenylalanine racemase, an enzyme catalysing the interconversion of L-phenylalanine and D-phenylalanine using ATP as a cofactor. As usual for a racemase (see cluster F), this reaction has a C(R/S) stereoechange as an attribute, however it also shares the presence of O–P and H–O with ten intramolecular transferase reactions (EC 5.4). Although the usage of ATP makes this enzyme unique among isomerases, its bond changes place it between two subclasses: racemases and epimerases (EC 5.1) and intramolecular transferases.
EC 5.3.3.7 is located in cluster B and corresponds to aconitate isomerase, which catalyses the transformation of trans-aconitate and cis-aconitate. It shares the presence of C(E/Z) with eight cis-trans isomerase reactions (EC 5.2). According to bond changes only, this biochemical transformation is closer to a cis-trans isomerase reaction than to an intramolecular oxidoreductase.

Finally, EC 5.2.1.5 is in cluster C and corresponds to linoleate isomerase and catalyses the interconversion of linoleate and 9-cis,11-trans-octadecadienoate. As most cis-trans isomerases (EC 5.2) (cluster B), it has a bond change C(E/Z) as chemical attribute, however it also shares identical bond changes to three intramolecular oxidoreductase reactions (EC 5.3), namely EC 5.3.3.8, 5.3.3.13 and 5.3.3.14.

These results suggest that only 6 clusters are enough to separate all isomerase reactions. However only clusters B and F purely correspond to subclasses EC 5.1 and 5.2. The other clusters are mixtures. Although the choice of clustering algorithm and number of clusters was optimal in terms of fitting EC subclasses, this choice was verified by visual inspection. A lower or higher number of clusters or different clustering parameters at the expense of a more coarse or fine-grained description of the clusters could have been selected. However it might not have aligned to the EC classification of isomerases as closely as this procedure.

To summarise, from the similarity and clustering by bond changes subclasses EC 5.1 and 5.2 stand out from the rest as having distinct distinct bond change attributes - only C(R/S) or C(E/Z) stereochanges, respectively - which distinguish them from the rest. The other subclasses involve more complex combinations of bond changes.

### 3.2 Analysis of reaction centres

Using a similar strategy as in the analysis of bond changes, this section explores the distribution, similarity and clustering of isomerase reactions on the basis of reaction centres. Results on whether isomerase EC subclasses are better described by reaction centres rather than just by bond changes is presented in 3.4 Discussion.

#### 3.2.1 Distribution

There are 6354 occurrences of 595 distinct reaction centres (levels 0, 1 and 2) in isomerase reactions (for details about calculating reaction centres see 2.2.2 Methods and tools). As observed in the distribution of bond changes, the six isomerase subclasses have different
numbers of reactions centres (Figure 3.6a). Intramolecular transferases (EC 5.4), followed by intramolecular oxidoreductases (EC 5.3) are the most populous subclasses by means of reaction centres. Then, medium-size subclasses are intramolecular lyases (EC 5.5) accounting for 666 reaction centres and racemases and epimerases (EC 5.1) with 420 reaction centres. Ultimately, cis-trans isomerases (EC 5.2) and other isomerases (EC 5.99) have a smaller number of reaction centres.

Figure 3.6: Distribution of reaction centres in isomerase reactions. (a) Distribution of the 6354 occurrences of reaction centres in 219 representative isomerase reactions across EC subclasses. (b) Bar plot showing the number of isomerase reactions against number of reaction centres. (c) Box and whisker plot representing the distribution of the number of reaction centres per isomerase reaction by EC subclass.

There is a clear distinction between EC 5.1, EC 5.2 and the rest (Figure 3.6b), as observed for bond changes. The number of reaction centres per isomerase reaction ranges from 6 to 132. It is by definition always an even number due to the atom-atom mapping between the substrates and products of the reaction. The average number of reaction centres per isomerase reaction is 29 and there are a few extreme intramolecular transferases (EC 5.4)
with 114, 120 and 132 reaction centres. In general, racemases and epimerases (EC 5.1) and cis-trans isomerases (EC 5.2) catalyse either 6 or 12 reaction centres, whereas the rest of subclasses range from 18 to 132 (see Figure 3.6c).

Figure 3.7: Distribution of isomerase reactions according to the 30 most common reaction centres.

Reaction centres were named using IUPAC nomenclature (IUPAC, 2014) and the most common are carbon atom, oxygen atom and 2-hydroxypropyl (Figure 3.7). Subclasses EC 5.1, 5.2 and 5.99 are connected with up to six distinct reaction centres among the most common ones. For example, EC 5.2 relates to six of them: carbon atom, propyl, prop-2-en-1-yl, 2-methylprop-2-en-1-yl, prop-1-en-1-ylidene and 2-methylprop-1-en-1-ylidene. The rest of subclasses have many of the most common reaction centres, which underlines the broad chemical diversity of these classes. For instance, all of the top 30 reaction centres are present in EC 5.4. On the whole, the top 22 reaction centres (up to 2,2-dimethylpropyl in Figure 3.7) are present in at least three subclasses, highlighting how
enzymes from different subclasses can perform catalysis on the same reaction centre. As in the analysis of bond changes, subclasses EC 5.1 and 5.2 have less diversity and frequency of reaction centres compared to the other subclasses.

When calculating the degree to which reaction centres are specific to certain subclasses, we discovered that rare reaction centres, for instance (3,3-dimethyloxiran-2-yl)methyl (Figure 3.7), are only present in one subclass (SW=1). On the other hand, abundant reaction centres, such as 2-hydroxypropyl are distributed across more than one subclass (SW<1). In the former, there are 25 enzymes classified as intramolecular transferases acting on squalene derivatives (EC 5.4.99), which are active on (3,3-dimethyloxiran-2-yl)methyl as a reaction centre, for example, lanosterol synthase (EC 5.4.99.7). However in the latter, there are 102 isomerases from four different subclasses: EC 5.1, 5.3, 5.4, 5.5 catalysing isomerase reactions where 2-hydroxypropyl is a reaction centre. Overall, 468 reaction centres (79% of the total) are perfect discriminators of subclasses and some of them are present in multiple isomerase reactions of the same subclass.

3.2.2 Similarity and clustering

The distribution of pairwise similarities of isomerase reactions based on reaction centres has a different shape to the bond change distribution. Whereas bond change similarity scores adopt a reverse J-shaped distribution with three peaks at similarities 0, 0.15 and 1 (Figure 3.4a), reaction centres follow a normal distribution with positive skew (Figure 3.8a) and mean similarity of 0.22 (bond changes mean similarity is 0.20). The complete-linkage algorithm was selected to produce an optimal number of 10 clusters (Figure 3.8b and 3.9), which are summarised in Table 3.3.

Seven clusters are pure in subclasses (B’, C’, D’, G’, H’, I’ and J’), although three of them are singletons (D’, H’ and J’). The three remaining clusters are mixed. All subclasses are spread across more than one cluster. Racemases and epimerases (EC 5.1) is the subclass adopting the best clustering where 98% of reactions are placed in pure clusters whereas the rest are in mixed clusters. However, intramolecular oxidoreductases (EC 5.3) have most reactions assigned to mixed clusters.

Almost all the reactions (216 out of 219) have at least one carbon atom involved in bond changes. The three exceptions are the intramolecular transfer reactions catalysed by bisphosphoglycerate mutase (EC 5.4.2.4) and 2,3-diphosphoglycerate-dependent and independent phosphoglycerate mutase (EC 5.4.2.11 and 5.4.2.12) (see cluster G’ in Figure 3.9), which catalyse the cleavage of O–P and O–H bonds only. This analysis discovered 11 reactions with distinct profiles of reaction centres (Table 3.3). For instance,
Figure 3.8: (a) Distribution of pairwise similarities of isomerase reactions by reaction centres. (b) Reaction centre similarity matrix.
Figure 3.9: Hierarchical clustering of isomerase reactions (rows) by reaction centres (columns). Ten clusters were identified (A' to J'). Only the 30 most common reaction centres are shown.
Table 3.3: Analysis of the clusters from Figure 3.9. Columns are defined as in the bond changes analysis (Table 3.2). Cluster names are arbitrary. Clusters A’ to J’ obtained by reaction centres do not necessarily correspond to clusters A to F obtained by bond changes.

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Number of reactions</th>
<th>EC subclasses</th>
<th>Most common reaction centres</th>
<th>Outliers and comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>A’</td>
<td>38</td>
<td>EC 5.2 (4), 5.3 (12), 5.4 (19), 5.5 (2) and 5.99 (1)</td>
<td>carbon atom and propyl</td>
<td>EC 5.5.1.19, 5.2.1.15 and 5.3.3.7 are distinct reactions. Mixed cluster</td>
</tr>
<tr>
<td>B’</td>
<td>5</td>
<td>EC 5.2 (5)</td>
<td>carbon atom and prop-2-en-1-yl</td>
<td>No outliers. Pure cluster</td>
</tr>
<tr>
<td>C’</td>
<td>56</td>
<td>EC 5.1 (56)</td>
<td>carbon atom, 2-hydroxypropyl and 2-azanylidenepropyl</td>
<td>No outliers. Pure cluster</td>
</tr>
<tr>
<td>D’</td>
<td>1</td>
<td>EC 5.1 (1)</td>
<td>carbon atom</td>
<td>No outliers. EC 5.1.3.24 is a singleton</td>
</tr>
<tr>
<td>E’</td>
<td>56</td>
<td>EC 5.3 (40), 5.4 (9), 5.5 (6) and 5.99 (1)</td>
<td>carbon atom, oxygen atom, 2-hydroxypropyl, hydroxymethyl, oxomethylidene and 2-oxopropyl</td>
<td>EC 5.5.1.23, 5.4.2.9 and 5.5.1.5 are distinct reactions. Mixed cluster</td>
</tr>
<tr>
<td>F’</td>
<td>46</td>
<td>EC 5.2 (1), 5.3 (4), 5.4 (30) and 5.5 (11)</td>
<td>Most of the top 30 reaction centres</td>
<td>EC 5.5.1.20, 5.5.1.18 and 5.4.99.38 are distinct reactions. Mixed cluster</td>
</tr>
<tr>
<td>G’</td>
<td>12</td>
<td>EC 5.1 (1), 5.3 (1) and 5.4 (10)</td>
<td>Oxygen atom, hydroxymethyl and 2-hydroxyethyl</td>
<td>EC 5.1.1.11 and 5.3.2.5 are distinct reactions. Almost pure cluster.</td>
</tr>
<tr>
<td>H’</td>
<td>1</td>
<td>EC 5.5 (1)</td>
<td>Carbon atom, oxygen atom and oxomethylidene</td>
<td>No outliers. EC 5.5.1.3 is a singleton</td>
</tr>
<tr>
<td>I’</td>
<td>3</td>
<td>EC 5.4 (3)</td>
<td>-</td>
<td>No outliers. Pure cluster</td>
</tr>
<tr>
<td>J’</td>
<td>1</td>
<td>EC 5.2 (1)</td>
<td>-</td>
<td>No outliers. EC 5.2.1.8 is a singleton</td>
</tr>
</tbody>
</table>
situated in cluster A’ aconitate isomerase (EC 5.3.3.7) shares at least two common reaction centres (carbon atom and 2-methylprop-2-en-1-yl) with furylfuramide isomerase (EC 5.2.1.6), beta-carotene isomerase (EC 5.2.1.14) and other cis-trans isomerases (EC 5.2) (see cluster B’ in Figure 3.9) while remains distinct due to the presence of prop-2-en-1-yl and absence of prop-1-en-1-ylidene.

Although the analysis by bond changes identified three reactions that appear to be more similar to other isomerase subclasses and were labeled as misannotated (EC 5.1.1.11, 5.2.1.5 and 5.3.3.7), these have distinct profiles of reaction centres. Similarly, the two reactions annotated as distinct by bond changes remain distinct (EC 5.4.99.38) and turned into a singleton (EC 5.5.1.3) by reaction centres. The clustering by reaction centres provides a more detailed view of the overall chemistry of isomerases and helps to resolve most of the discrepancies found in bond changes.

Overall, this analysis of reaction centres supports the drastic difference in overall chemistry between EC 5.1 and 5.2 and the rest of subclasses as presented in the analysis of bond changes. Whereas reactions belonging to EC 5.1 and 5.2 contain only a few simple reaction centres such as 2-hydroxypropyl, 2-azanylidenepropyl and prop-2-en-1-yl, the rest of subclasses undergo more complex combinations of reaction centres highlighting the chemical diversity catalysed by these enzymes.

### 3.3 Analysis of substrates and products

The third chemical attribute used to investigate the functional similarity between isomerases is the structures of the substrates and products of the reactions. KEGG assumes all isomerase reactions to be reversible. Although this might not be true in all cases, reversibility was also accepted in this analysis so both substrates and products were broadly designated as reactants.

#### 3.3.1 Distribution

A total of 442 occurrences of 370 unique substrates and products are present in isomerase reactions. Almost all reactions are unimolecular (a single substrate leads to a single product), the only exception is the interconversion between L-phenylalanine and D-phenylalanine catalysed by L-phenylalanine racemase (EC 5.1.1.11), which is an ATP-hydrolysing isomerase and involves three substrates (L-phenylalanine, ATP and water) and three products (D-phenylalanine, AMP and diphosphate). As a result, the number of substrates and products in an isomerase subclass is about double the number of reactions. The distributions of substrates and products per reaction are proportionally
Figure 3.10: Distribution of substrates and products in isomerase reactions. (a) Distribution of the 442 occurrences of substrates and products in 219 representative isomerase reactions across EC subclasses (b) Bar plot showing the distribution of the 30 most common compounds present as substrates or products in isomerase reactions.
similar across subclasses (Figure 3.10a). Intramolecular transferases (EC 5.4), followed by racemases and epimerases (EC 5.1) and intramolecular oxidoreductases (EC 5.3) are the subclasses containing more substrates and products. With a much smaller number of reactions, intramolecular lyases (EC 5.5), cis-trans isomerases (EC 5.2) and other isomerases (EC 5.99) involve few substrates and products.

Almost 10% of the substrates and products are present in more than one reaction. The three most common substrates and products are: (S)-2,3-epoxysqualene, geranylgeranyl diphosphate and prostaglandin H2 and each exists in only one subclass: EC 5.4, 5.5 and 5.3, respectively (Figure 3.10b). Remarkably, (S)-2,3-epoxysqualene, an intermediate in the biosynthesis of terpenoids in plants, animals and fungi, is the substrate of 25 different oxidosqualene cyclases (EC 5.4.99.-), which catalyse diverse cyclisation/rearrangement reactions to produce cyclic sterols and triterpenes products (Abe, 2014). Particularly, these intramolecular transferases differ minimally in the structure of their active sites to generate structurally diverse cyclisation products. Geranylgeranyl diphosphate is also involved in cyclisation reactions undertaken by 5 different intramolecular lyases (EC 5.5.1.-) present in the mevalonate pathway of higher eukaryotes and bacteria. As for most prostaglandins, prostaglandin H2 is a lipid metabolite functioning as an important regulatory molecule in animals. It is the substrate of 4 different intramolecular oxidoreductases (EC 5.3.99.-), which share similar patterns of bond changes (Figure 3.5) and reaction centres (Figure 3.9). In contrast, the other most common substrates and products are reactants of enzymes from different subclasses. For instance, D-glucose 6-phosphate, the second metabolite of the glycolysis pathway, is the substrate of 4 isomerases from 3 different subclasses: phosphoglucose isomerase (EC 5.3.1.9), phosphoglucomutase (EC 5.4.2.2 and 5.4.2.5) and 1D-myo-inositol-3-phosphate lyase (EC 5.5.1.4).

3.3.2 Similarity and clustering

As in the analysis of reaction centres, the distribution of reaction similarities based on the structure of substrates and products adopts a normal distribution with positive skew (Figure 3.11a). Structural similarities are greater than similarities based on reaction centres with a mean similarity of 0.32. The best fit to isomerase subclasses was obtained using the complete-linkage algorithm in the form of five optimal clusters (Figures 3.11b and 3.12) summarised in Table 3.4.

All five clusters are mixed containing reactions from two or more subclasses. Racemases and epimerases (EC 5.1) and intramolecular transferases (EC 5.4) are present in all clusters. Intramolecular oxidoreductases (EC 5.3), cis-trans isomerases (EC 5.2) and
Figure 3.11: (a) Distribution of pairwise similarities of isomerase reactions by the structure of substrates and products. (b) Structure of substrates and products similarity matrix.
Figure 3.12: Hierarchical clustering of similarities of isomerase reactions (rows) based on the structures of substrates and products (columns). Five clusters were identified (A'' to E''). Only the 30 most common reaction substrates and products are shown.
intramolecular lyases (EC 5.5) exist in four, three and two clusters, respectively. Finally, other isomerases (EC 5.99) are only present in cluster B”. More than 90% of the substrates and products are just involved in one isomerase reaction, therefore analysing outliers (distinct and misannotated reactions) is not helpful here.

Table 3.4: Analysis of the clusters from Figure 3.12. Columns are defined as in the bond changes and reaction centres analyses (Tables 3.2 and 3.3).

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Number of reactions</th>
<th>EC subclasses</th>
<th>Common substrates and products</th>
</tr>
</thead>
<tbody>
<tr>
<td>A”</td>
<td>74</td>
<td>EC 5.1 (9), 5.2 (3), 5.3 (33), 5.4 (14) and 5.5 (15)</td>
<td>Geranylgeranyl diphosphate, D-glucose 6-phosphate and prostaglandin H2</td>
</tr>
<tr>
<td>B”</td>
<td>43</td>
<td>EC 5.1 (2), 5.2 (1), 5.3 (6), 5.4 (27), 5.5 (5) and 5.99 (2)</td>
<td>(S)-2,3-epoxysqualene</td>
</tr>
<tr>
<td>C”</td>
<td>49</td>
<td>EC 5.1 (29), 5.3 (8) and 5.4 (12)</td>
<td>dTDP-4-oxo-6-deoxy-D-glucose</td>
</tr>
<tr>
<td>D”</td>
<td>28</td>
<td>EC 5.1 (4), 5.2 (7), 5.3 (10) and 5.4 (7)</td>
<td>Methylitaconate and squalene</td>
</tr>
<tr>
<td>E”</td>
<td>25</td>
<td>EC 5.1 (14) and 5.4 (11)</td>
<td>L-glutamate and L-lysine</td>
</tr>
</tbody>
</table>

Although the analysis of bond changes and reaction centres revealed a clear difference in the overall chemistry of EC 5.1, 5.2 and the rest of subclasses, this distinction is not observed in the analysis of substrates and products. However this study is useful to discover similar isomerase reactions from different subclasses based on the structure of their reactants. For instance, in cluster E”, lysine racemase (EC 5.1.1.5) is adjacent to lysine 2,3-mutase (EC 5.4.3.2) and methylornithine synthase (EC 5.4.99.58). The first isomerase catalyses the racemisation of L-lysine to D-lysine. The second is a radical S-adenosyl-L-methionine (SAM) enzyme and transfers an amino group from C2 to C3 in L-lysine to produce (3S)-3,6-diaminohexanoate (Frey et al., 2008). Finally, the third is also a SAM enzyme catalysing a mutase reaction that uses L-lysine to generate 3-methylornithine, a key precursor in the biosynthesis of pyrrolysine, the twenty-second proteinogenic amino acid encoded as the UAG codon in the genetic code of methanogenic archaea and bacteria (Gaston et al., 2011). Although UAG is the stop codon in the standard genetic code, it is an exception in these organisms. Although the three enzymes use L-lysine as a substrate and also, the products are structurally similar, there are differences in their overall chemistry. The two SAM enzymes share similar chemistry as evidenced by bond changes and reaction centres, however the chemistry of lysine racemase is different.

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3.4 Discussion

3.4.1 Overall

This chapter explores chemical attributes (bond changes, reaction centres and structures of substrates and products) of all known isomerase reactions, which were then utilised to calculate similarity between reactions using EC-BLAST and clustering of reactions into groups of similar chemistry. Although previous studies found overall agreement between clustering of biochemical reactions and the EC classification, especially in oxidoreductase (EC 1), hydrolase (EC 3) and ligase (EC 6) reactions (Egelhofer et al., 2010; Holliday et al., 2014; Hu et al., 2010; Sacher et al., 2009), this analysis demonstrates that isomerase reactions are chemically diverse and challenging to classify using a hierarchical system. Other approaches found difficulties to handle isomerase reactions because they often involve stereochanges in the structures of substrates and products only, so no bonds are formed, cleaved or order changed from an overall perspective (Apostolakis et al., 2008; Chen et al., 2013; Körner & Apostolakis, 2008; Latino et al., 2008).

Reaction similarity and clusters obtained using bond changes are more similar to results obtained using reaction centres than those obtained using substrates and products. First, although the similarity distributions (Figures 3.4a, 3.8a and 3.11a) are all significantly different according to the Kolmogorov-Smirnov test (p<0.001), there is higher correlation between bond changes and reaction centres (Pearson’s correlation coefficients (r) = 0.51, p<0.001) than between bond changes and substrates and products (r = 0.21, p<0.001) (Figure 3.13) (Oksanen, 2011; R Core Team, 2012). Second, clusters of isomerase reactions by bond changes are more similar to clusters by reaction centres than to clusters by substrates and products (Figure 3.14). Topological distances between clustering trees also confirm this observation. For example, bond change clusters A (11 reactions), D (20 reactions), E (37 reactions) and F (56 reactions) directly correspond to reaction centre clusters G’, E’, F’ and C’, respectively. However there is not a clear correspondence between clusters by bond changes and substrates and products.

This analysis presents however some clear caveats. Although mechanistic steps and cofactors are essential to understand the chemistry of isomerases and enzymes in general (Holliday et al., 2009), this study ignores these aspects by relying upon the overall chemistry of reactions only. There are three main reasons for this. First, mechanistic components are not captured in reaction files (except cofactors such as ATP and NADH, which are sometimes included). Second, mechanistic details are difficult to retrieve from literature and are scarce in databases. For instance, at the time of writing only one-fifth (43) of the isomerase reactions have mechanistic data in the development version of MA-
Figure 3.13: Comparison of similarity distributions of isomerase reactions as calculated by bond changes, reaction centres and substrates and products. Each point represents a pair of isomerase reactions.
Figure 3.14: Comparison of clusters of isomerase reactions as calculated by bond changes, reaction centres and substrates and products using contingency tables (left) and tanglegrams (right) (Scornavacca et al., 2011).

CiE. Third, a recent study showed that overall reaction information performs better at predicting isomerase EC numbers than mechanistic descriptors (Nath & Mitchell, 2012). Nevertheless there are still some isomerase reactions involving cyclisations of (S)-2,3-epoxysqualene and derivatives (EC 5.4.99) which are fairly complex even at the overall level. As a result, these reactions are challenging for atom-atom mapping and difficult to handle in EC-BLAST. This led to a likely overestimation of their number of bond changes (Figure 3.2a), which will be addressed in future releases of EC-BLAST.
3.4.2 Correspondence with isomerase EC subclass

The overall statistics of bond changes, reaction centres and substrates and products found in isomerase reactions are displayed in Table 3.5. Bond changes are best for partially recreating the current EC classification of isomerase reactions into six subclasses. Nevertheless, the bond change distribution is not pure, which reflects the complex, surely non-hierarchical, nature of the chemistry of isomerases. Reaction centres generate a more complex classification but support the results obtained by bond changes. Structures of substrates and products are not helpful for the EC classification but are useful to find enzymes which work on the same reactants.

Table 3.5: Table displaying statistics of the chemical attributes found in isomerase reactions. Column Types refers to the number of distinct types of chemical attributes. Columns Pairs (total, observed and frequency) represent the total number of possible pairs (Types × 219), observed pairs and frequency of observed pairs of attributes and reactions, respectively. Column specificity indicates the number and proportion of attributes specific to an isomerase subclass (see main text).

<table>
<thead>
<tr>
<th>Chemical attribute</th>
<th>Types</th>
<th>Pairs (total)</th>
<th>Pairs (observed)</th>
<th>Pairs (frequency)</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bond changes</td>
<td>30</td>
<td>6570</td>
<td>746 (11.3%)</td>
<td>1603</td>
<td>12 (40%)</td>
</tr>
<tr>
<td>Reaction centres</td>
<td>595</td>
<td>139065</td>
<td>3067 (2.2%)</td>
<td>6354</td>
<td>468 (79%)</td>
</tr>
<tr>
<td>Substrates and products</td>
<td>370</td>
<td>81030</td>
<td>442 (0.5%)</td>
<td>442</td>
<td>354 (96%)</td>
</tr>
</tbody>
</table>

The specificity of chemical attributes to isomerase EC subclasses (Table 3.5) is not a fundamental property of the subclass but depends on the chemical nature of reactions and their subclass association as established by the NC-IUBMB. Although specificity increases from bond changes (40%) to reaction centres (79%) to substrates and products (96%), the latter are rare given the small proportion (0.5%) of possible unique combinations that are observed. The ability of the NC-IUBMB to manually update the EC classification in the form of new, transferred and deleted reactions as new enzyme data becomes available will change the specificity of chemical attributes for subclasses. In line with this, the analysis of bond changes first highlighted potential inconsistencies for three isomerase EC numbers, which were later recognised as distinct reactions in the analysis of reaction centres.

Isomerases are a rare class of enzymes. Unlike other EC classes such as the ligases (EC 6) (Holliday et al., 2014), their functional classification is rather complex. First, the frequency of bond changes and reaction centres is drastically different between subclasses. Racemases and epimerases and cis-trans isomerases (EC 5.1 and 5.2) have relatively few bond changes and reaction centres compared to intramolecular oxidoreductases, in-
tramolecular transferases and intramolecular lyases (EC 5.3, 5.4 and 5.5). Second, the overall chemistry differs significantly between subclasses as well. While EC 5.1 and 5.2 are sensibly grouped according to changes of stereochemistry (clusters B and F in Figure 3.5 and clusters A’, B’ and C’ in Figure 3.9), EC 5.3, 5.4 and 5.5 are present in mixed clusters and are also very similar in overall chemistry to other EC primary classes, oxidoreductases (EC 1), transferases (EC 2) and lyases (EC 4), respectively. The subclass “other isomerases” (EC 5.99) sits apart from the rest and exhibits great diversity, as evidenced by the distinct chemistry of winding DNA catalysed by topoisomerases (O’Brien, 2006).

The overall chemistry of isomerases does not always involve stereochanges (C(R/S) or C(E/Z)), there are 45 isomerase reactions that do not have any stereochange (Figure 3.5). According to the NC-IUBMB, isomerases catalyse structural rearrangements between isomers, namely the substrate and product have the same molecular formula but different chemical structures. This definition obviates the need to know whether reactions undergo stereochanges. For example, isopentenyl-diphosphate ∆-isomerase (EC 5.3.3.2) interconverts two isomers: isopentenyl diphosphate and dimethylallyl diphosphate, but no stereochange takes place (see Figure 2.3 from Chapter 2. Data Resources and Methods). The first step of the reaction is protonation of a C=C bond and formation of a carbocation, and then reformation of the C=C bond by deprotonation of an adjacent carbon (MACiE mechanism M0190 (Holliday et al., 2012)).

The EC classification of isomerases in six subclasses can effectively be simplified according to the IUPAC definitions of isomers (IUPAC, 2014). There are two types:

(a) **Stereoisomers**: molecules only differ on the spatial location of atoms without any differences in atom connectivity. The substrate and product of enzymes belonging to subclasses EC 5.1 and 5.2 are stereoisomers. As a result, the only chemical attributes are stereochanges such as R/S and E/Z isomerisations.

(b) **Structural isomers**: molecules differ in atom connectivity, which as a result changes the spatial location of atoms as well. The substrate and product of enzymes belonging to subclasses EC 5.3, 5.4 and 5.5 are structural isomers. Chemical attributes are not only stereochanges, but also formed/cleaved bonds and bond order changes.

The clear separation between EC 5.1 and 5.2 and the rest of isomerase subclasses observed in the analysis of bond changes and reaction centres suggests that classifying isomerases in two subclasses according to the type of isomerism between substrate and product is chemically more sensible than the current classification in six subclasses. This recommendation involves the reorganisation of isomerases in two groups: stereoisomerases (current
EC 5.1 and 5.2) and structural isomerases (current EC 5.3, 5.4 and 5.5). Regarding EC 5.99, topoisomerases (EC 5.99.1.2 and 5.99.1.3) change the topology of DNA. Although the overall chemistry of these enzymes is not described by any descriptor, they qualify as stereoisomerases because they change the spatial location of atoms while maintaining the atom connectivity. In addition, their catalytic mechanism involve catalysis of O–P bonds in the phosphate backbone of DNA (MACiE entries M0064, M0232 and M0366). Finally, thiocyanate isomerase (EC 5.99.1.1) and 2-hydroxychromene-2-carboxylate isomerase (EC 5.99.1.4) qualify as structural isomerases.

### 3.4.3 Correspondence with isomerase EC sub-subclass

Only the subclasses racemases and epimerases (EC 5.1), intramolecular oxidoreductases (EC 5.3) and intramolecular transferases (EC 5.4) are further divided into sub-subclasses (Figure 3.15 and Table 3.6).

Table 3.6: Statistics of the chemical attributes of the isomerase subclasses that split into sub-subclasses: racemase and epimerase (EC 5.1), intramolecular oxidoreductase (EC 5.3) and intramolecular transferase (EC 5.4) reactions.

<table>
<thead>
<tr>
<th></th>
<th>EC 5.1</th>
<th>EC 5.3</th>
<th>EC 5.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactions</td>
<td>58</td>
<td>57</td>
<td>71</td>
</tr>
<tr>
<td>Bond changes</td>
<td>3</td>
<td>16</td>
<td>22</td>
</tr>
<tr>
<td>Reaction centres</td>
<td>61</td>
<td>233</td>
<td>309</td>
</tr>
<tr>
<td>Substrates and products</td>
<td>115</td>
<td>104</td>
<td>108</td>
</tr>
</tbody>
</table>

**Racemase and epimerase reactions (EC 5.1)**

Fifty-eight racemases and epimerases catalyse chiral inversions between stereoisomers, also known as changes in absolute stereochemistry (R or S) of asymmetric carbon atoms. This subclass is divided into 4 sub-subclasses depending on the nature of the substrate. Racemases from EC 5.1.1 act on amino acids and derivatives, EC 5.1.2 on hydroxy acids and derivatives, EC 5.1.3 on carbohydrates and derivatives and EC 5.1.99 on “other compounds”. The analysis of bond changes is simple, only three bond changes are involved, all EC 5.1 reactions have only C(R/S) with the exception of phenylalanine racemase (EC 5.1.1.11), which also catalyses O–P and O–H. The similarity and clustering analyses based on the 61 unique reaction centres and the 115 substrates and products suggest two complementary ways to explore these sub-subclasses (Figure 3.16).

Both approaches set EC 5.1.1 and 5.1.3 reactions apart from each other in two different clusters. EC 5.1.1 reactions are characterised by 2-azanylidenepropyl as the most common reaction centre in amino acids as reactants. In contrast, EC 5.1.3 reactions are characterised by the reaction centre 2-hydroxypropyl in sugars and derivatives. However,
there are also reactions sharing characteristic attributes of the two sub-subclasses. For instance, epimerases acting on glucosamine and derivatives (EC 5.1.3.8, 5.1.3.9, 5.1.3.14 and 5.1.3.23) catalyse transformations of the reaction centre 2-azanylidenepropyl not in amino acids but in sugar molecules. Alternatively, threonine racemase (EC 5.1.1.6) acts on the amino acid threonine even though 2-hydroxypropyl is also a reaction centre. In addition, amino acid racemase (EC 5.1.1.10) and hydantoin racemase (EC 5.1.99.5) form a separate cluster in Figure 3.16a because they catalyse reactions containing a generic substructure or R-group in the reaction centre (for a detailed explanation of R-groups see Chapter 5. Characterising Complex Biochemical Reaction Data).

Although EC 5.1.2 reactions group with EC 5.1.3 due to the presence of the 2-hydroxypropyl as reaction centre (Figure 3.16a), they cluster with EC 5.1.1 reactions in substrates and products because hydroxy acids resemble amino acids more than carbohydrates at
To summarise, this analysis shows a clear separation between the overall chemistry of EC 5.1.1 and 5.1.3 reactions. The results are consistent with previous investigations of the overall chemistry of EC 5.1 reactions using a different strategy based on chirality codes and self-organising maps (Latino et al., 2008). EC 5.1.2 and 5.1.99 reactions are diverse and group alternately with EC 5.1.1 and 5.1.3.
**Intramolecular oxidoreductase reactions (EC 5.3)**

The larger number of chemical attributes of the 57 intramolecular oxidoreductases (EC 5.3) indicates a more diverse chemistry in comparison with EC 5.1 reactions. This subclass is divided in 5 sub-subclasses. EC 5.3.1 interconvert aldoses and ketoses, EC 5.3.2 transform keto and enol groups, EC 5.3.3 and 5.3.4 transpose C=C and S–S bonds, respectively, and EC 5.3.99 act on other substrates. This subclass is characterised by 16 bond changes, 233 reaction centres and 104 substrates and products (Table 3.6). Subsubclass EC 5.3.4 contains only one reaction catalysed by protein disulfide-isomerase (EC 5.3.4.1). The substrate of this enzyme is a protein, which does not have a structure file available, therefore it is not considered in this study.

![Figure 3.17: Hierarchical clustering of intramolecular oxidoreductase reactions (rows) based on (a) bond changes, (b) reaction centres and (c) structures of substrates and products (columns). Only the thirty most common chemical attributes are shown in (b) and (c). Clusters are indicated in boxes and reactions discussed in the main text are marked with arrows.](image)

Overall, cluster analysis indicates a clear distinction between the overall chemistry of EC 5.3.1 and 5.3.3 reactions (Figure 3.17). Despite EC 5.3.2 reactions clustering with EC 5.3.1 and 5.3.3 in the analysis of bond changes, they solely group with EC 5.3.1 according
to reaction centres but with EC 5.3.3 in substrates and products. For example, the reactions catalysed by TDP-6-deoxy-hex-4-ulose isomerase (EC 5.3.2.3) and TDP-4-oxo-6-deoxy-alpha-D-glucose-3,4-oxoisomerase (EC 5.3.2.4) share similar bond changes and reaction centres with EC 5.3.1 reactions, however the overall structure of their reactants resemble EC 5.3.3 reactions more closely. Similarly, the reaction catalysed by trans-2,3-dihydro-3-hydroxyanthranilate isomerase (EC 5.3.3.17) shares H–O and C–O ↔ C=O with EC 5.3.2 reactions. Finally, EC 5.3.99 reactions group with 5.3.1 and 5.3.3 in bond changes but form separate clusters in reaction centres and substrates and products. For instance, thiazole tautomerase (EC 5.3.99.10) catalyses a C=C transposition, which is the most common bond change in EC 5.3.3 reactions. However, prostaglandin A isomerase (EC 5.3.3.9) also catalyses a C=C transposition, but its substrate is (13E)-(15S)-15-Hydroxy-9-oxoprosta-10,13-dienoate, which is structurally similar to prostaglandin H2, a common substrate in EC 5.3.99.

Intramolecular transferase reactions (EC 5.4)

The 71 intramolecular transferase reactions (EC 5.4) are grouped into 5 sub-subclasses depending on the nature of the transferred chemical group. EC 5.4.1 involve acyl groups, EC 5.4.2 transfer phospho groups, EC 5.4.3 shift amino groups, EC 5.4.4 involve hydroxy groups and last but not least, EC 5.4.99 transfer other groups. For instance, isochorismate synthase (EC 5.4.4.2) and chorismate mutase (EC 5.4.99.5) isomerise the substrate chorismate into isochorismate and prephenate, respectively. Although both reactions share the same substrate and similar bond changes and reaction centres, the former involves the transfer of a hydroxy group whereas the latter converts a 2-hydroxyprop-2-enoic acid group. The subclass EC 5.4 is dominated by the sub-subclass EC 5.4.99 where 44 reactions account for 62% of all EC 5.4 reactions and one-fifth of all EC 5 reactions, therefore defining EC 5.4.99 as the most populated sub-subclass in isomerases. Chemically speaking, different types of bond changes and reaction centres result in a complex and diverse overall chemistry, especially the cyclisation reactions catalysed by oxidosqualene cyclases (Figure 3.18).

EC 5.4.1 contains only one reaction catalysed by lysolecithin acylmutase (EC 5.4.1.1). EC 5.4.2 reactions involve O–P bonds and group together according to bond changes and reactions centres (Figure 3.18a,b), however in the analysis of substrates and products they split into two groups according to acyclic and cyclic reactants, especially sugar phosphates (Figure 3.18c). Interestingly, the reaction catalysed by phosphoenolpyruvate mutase (EC 5.4.2.9) sits apart from the rest of EC 5.4.2 reactions because it involves the formation of C–P bonds. This extra chemical ability has been extensively explored since it allows the biosynthesis of phosphonates in nature (Yu et al., 2013). EC 5.4.3 reactions
38, 39 and 40 of nearly all tRNAs and it is the only reaction with structural data available. Pseudouridine synthase (EC 5.4.99.12) isomerises the uridine residues at positions where modifications take place (Hamma & Ferré-D’Amaré, 2006). For example, tRNA pseudouridine38-40 synthase (EC 5.4.99.12) isomerises the uridine residues at positions 38, 39 and 40 of nearly all tRNAs and it is the only reaction with structural data available.

Figure 3.18: Hierarchical clustering of intramolecular transferase reactions (rows) based on (a) bond changes, (b) reaction centres and (c) structures of substrates and products (columns). Only the thirty most common chemical attributes are shown in (b) and (c). Clusters are indicated in black boxes, oxidosqualene cyclisations are highlighted in purple boxes and reactions discussed in the main text are marked with arrows.

These reactions involve C–N bonds and cluster by bond changes and reaction centres in a similar way that EC 5.4.2 reactions do, however the analysis of their substrates and products segregates them into acyclic and aromatic reactants. EC 5.4.4 are diverse and spread across multiple clusters. Finally, in order to dissect the complexity of EC 5.4.99 reactions, the clustering results suggest that splitting EC 5.4.99 into additional sub-subclasses would be sensible according to an overall chemistry point of view. First, the 25 oxidosqualene cyclisations (EC 5.4.99.X, X = 7, 8, 17, 31-37, 39-41 and 46-57) act on (S)-2,3-epoxysqualene and derivatives and their bond changes, reaction centres and substrates and products are different from EC 5.4.99 and the rest of the isomerase reactions. Second, the chemistry of the 16 RNA pseudouridine synthases (EC 5.4.99.X, X = 12, 19-30 and 42-45) also sets apart from the rest of the EC 5.4.99 reactions. These enzymes post-transcriptionally isomerise specific uridine residues to pseudouridine in RNA. They all share the same overall chemistry but differ in the type of RNA (tRNA or rRNA) and the sequence sites where modifications take place (Hamma & Ferré-D’Amaré, 2006). For example, tRNA pseudouridine38-40 synthase (EC 5.4.99.12) isomerises the uridine residues at positions 38, 39 and 40 of nearly all tRNAs and it is the only reaction with structural data available.
in this analysis (Figure 3.18). Third, there are 6 mutases catalysing carbon rearrange-
ments (EC 5.4.99.X, X = 1, 2, 4, 13, 14 and 18), which also share similar overall chemistry. 
Overall, these three distinct groups of reactions within EC 5.4.99 could sensibly be con-
sidered as three new sub-subclasses.

3.5 Conclusion

The EC classification of isomerases is not easy. The various criteria used to define EC 
subclasses and sub-subclasses do not entirely follow a sensible classification based on 
chemical attributes of the overall reaction such as bond changes, reaction centres and 
substrates and products. This analysis suggests two main ways to improve the EC classi-
fication. First, the current classification of isomerases into six subclasses can be reduced 
to two subclasses according to the type of isomerism shared between substrate and prod-
uct. Racemases and epimerases (EC 5.1) and cis-trans isomerases (EC 5.2) group un-
der a “metaclass” entitled stereoisomerism whereas intramolecular oxidoreductases (EC 
5.3), intramolecular transferases (EC 5.4), intramolecular lyases (EC 5.5) and “other 
isomerases” (EC 5.99) represent structural isomerism. Second, complex sub-subclasses 
such as intramolecular transferases acting on “other groups” (EC 5.4.99) can be further 
split into groups of similar chemistry such as oxidosqualene cyclases and pseudouridine 
synthases in order to make the EC classification more useful.
Chapter 4

The Evolution of Isomerase Function

Almost thirty years ago, Chothia and Lesk started investigating the relationship between sequence and structural divergence in related proteins (Chothia & Lesk, 1986). A few years later, scientists began to explore the origins of the evolution of enzyme function using techniques from molecular biology, crystallography and enzymology (Petsko et al., 1993). Today, evidence suggests that the major route for creating new enzyme functions is gene duplication and subsequent evolution of one enzyme to another with a novel, though usually related, function (Copley, 2012). New computational approaches to measure functional similarity between enzymes extend the existing evolutionary studies based on sequence and structure. In this chapter our knowledge of the evolution of the isomerase class of reactions in enzyme superfamilies is reviewed, using newly developed tools to compare enzyme reactions (Rahman et al., 2014) and their evolution (Furnham et al., 2012b).

The study of protein superfamilies has revealed how enzymes evolve their overall chemistry and mechanism during evolution (Bartlett et al., 2003). Several groups have already attempted to explore the evolution of function in specific isomerases. Over the last two decades, comprehensive analyses of the enolase superfamily showed that mandelate racemase (EC 5.1.2.2) and muconate-lactonizing enzyme (EC 5.5.1.1) are related by divergent evolution from a common ancestor and they catalyse similar mechanisms but different overall chemistry (Gerlt et al., 2012; Petsko et al., 1993). Subsequent studies on sugar isomerases found that common ancestry, similar overall chemistry but different mechanisms is also an alternative evolutionary pathway (Banerjee et al., 1995). Recent experimental analyses presented evidence of how isomerase function exchanges with other EC classes (Table 4.1).
Table 4.1: Summary table of experimental studies exploring the evolution of isomerase function.

<table>
<thead>
<tr>
<th>Evolved from</th>
<th>Evolved to</th>
<th>Superfamily</th>
<th>Species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-succinylbenzoate synthases (EC 4.2.1.113)</td>
<td>N-succinylamino acid racemase (EC 5.1.1.1)</td>
<td>Enolase</td>
<td>*E. coli, B. subtilis, G. stearothermophilus and S. sp. PCC 6803</td>
<td>Glasner et al. (2006a)</td>
</tr>
<tr>
<td>Aspartate aminotransferase (EC 2.6.1.1)</td>
<td>Alanine, glutamate and aspartate racemases (EC 5.1.1.1, 5.1.1.13)</td>
<td>Pyridoxal 5’-phosphate-dependent enzymes</td>
<td><em>E. coli</em></td>
<td>O’Brien &amp; Herschlag (1999); Vacca et al. (1997)</td>
</tr>
<tr>
<td>Isochorismate pyruvate-lyase (EC 4.2.99.21)</td>
<td>Chorismate mutase (EC 5.4.99.5)</td>
<td>Chorismate mutase domain</td>
<td><em>P. aeruginosa</em></td>
<td>Künzler et al. (2005); Schulenburg &amp; Miller (2014)</td>
</tr>
<tr>
<td>Various hydrolases and lyases (EC 3 and 4)</td>
<td>Uronate isomerase (EC 5.3.1.12)</td>
<td>Amidohydrolase</td>
<td><em>E. coli and B. halodurans</em></td>
<td>Nguyen et al. (2008, 2009)</td>
</tr>
<tr>
<td>Maleylacetoacetate isomerase (EC 5.2.1.2)</td>
<td>TCHQ dehalogenase and 2,5-DCHQ dehalogenase (EC 1.97.1.)</td>
<td>Glutathione S-transferase</td>
<td><em>S. chlorophenolica</em></td>
<td>Anandarajah et al. (2000)</td>
</tr>
</tbody>
</table>

4.1 Availability of data on isomerase sequences and structures

In 24th July 2013, the NC-IUBMB listed 231 active four-digit isomerase EC numbers in the classification. Information about the enzyme sequences is easily accessible in UniprotKB (The Uniprot Consortium, 2013), 199 of them have sequence information and 32 are orphan isomerase EC numbers, also known as orphan enzymes (Lespinet & Labedan, 2005; Pouliot & Karp, 2007), a term given to EC numbers where no gene has been associated with these reactions and no sequence information is available in protein sequence repositories. Almost half of the isomerase EC numbers with sequence information (96) are present in FunTree and Figure 4.1a shows the distribution by EC 5 subclass.

Protein structural data are available for 126 isomerase EC numbers, which have at least
Figure 4.1: Distribution of isomerase data in sequence, structure, function and evolution resources. (a) Distribution of isomerases in EC classification, UniprotKB, PDB and FunTree. EC exchange matrices representing the changes in function during evolution of isomerases at the EC (b) class and (c) subclass levels. More frequent changes of isomerase function are highlighted in red. Green and blue boxes represent changes within isomerases and with other EC classes, respectively. (d) Frequency of EC changes involving isomerases by superfamily. The 32 superfamilies bearing multiple changes are illustrated.
one entry in the PDB (Berman et al., 2013). The 96 isomerases currently present in FunTree include domains, which are distributed across 81 CATH superfamilies: 17 are mostly alpha, 5 mostly beta and 59 mixed alpha/beta. Some superfamilies include more isomerases than others, for example, the superfamily UDP-galactose 4-epimerase, domain 1 (CATH 3.90.25.10) includes 7 racemases and epimerases (EC 5.1). In FunTree, one-third of the 96 isomerases include more than one domain superfamily (multidomain), with most of them including two or three superfamilies, but rarely more. Exceptionally, the subclass “other isomerases” (EC 5.99), which has two EC numbers (EC 5.99.1.2 and 5.99.1.3) is distributed across seven and eight superfamilies, respectively. These are types I and II DNA topoisomerases, which are characterised by multiple domains required for the complex process of winding DNA (O’Brien, 2006).

4.2 Observed changes of isomerase function

4.2.1 Change in EC number

Analysis of FunTree data on 58 domain superfamilies identified a total of 145 unique changes of isomerase activity that occurred during evolution (for details on how changes of function are calculated see 2.3.2 Methods and tools and Figure 2.8b). Only one-fifth of the changes occur between isomerases whereas the rest involve changing from isomerases to perform reactions in other EC primary classes (Figure 4.1b). This is strikingly different from enzymes in other EC classes where changes in lower levels of the EC classification are more common than changes in the primary classification (Furnham et al., 2012a). Among the 26 changes within isomerases, only 3 change the EC subclass and 23 change the EC serial number, indicating a change in substrate (Figure 4.1c). A previous limited study of 24 pairs of enzymes reported that changes involving isomerases and lyases (EC 5 ↔ EC 4) occur more often than changes to other EC classes (Bartlett et al., 2003). Other analyses provided further evidence of these changes by revealing the structural insights of the evolution of an isomerase from a family of lyases, namely N-succinylamino acid racemase (EC 5.1.1.-) from o-succinylbenzoate synthases (EC 4.2.1.113) in the enolase superfamily (Glasner et al., 2006a). This comprehensive analysis confirms that such changes are indeed prevalent, with 39% of the 119 changes in primary classification involving lyases.

Most domain superfamilies show multiple changes of reaction chemistry involving different EC classes (Figure 4.1d). The most adaptable superfamily domains are aldolase class I (CATH 3.20.20.70) and glutaredoxin (CATH 3.40.30.10), each of them exhibiting 10 changes of isomerase function. Whereas the glutaredoxin “isomerase” domain only exhibits changes of isomerase, oxidoreductase and transferase reactions, the aldolase class
I domain has also evolved to become a hydrolase and lyase (Figure 4.1d).

### 4.2.2 Correlation of sequence and function evolution

To gain an overview of the relationship between sequence and functional divergence, an overall representation of the sequence and functional similarity between the homologous enzymes that perform different catalytic reactions is presented in Figure 4.2. This illustrates that most sequences have diverged considerably, with sequence identities in the range lower than 40%. The three measures of functional similarity (Figure 4.2a-c) capture different properties of the change in function, but none of the plots show any linear relationship between sequence and functional divergence. In addition, the distributions for each of these measures look quite different. In Figure 4.2a, which assesses the overall bond changes, there are two clusters, one consists of changes exhibiting bond change conservation when the isomerase EC subclass is maintained, and in the second changes at the isomerase EC subclass or EC primary class do not exhibit bond change conservation. This partition is not observed in the comparisons by reaction centres and structures of substrates and products and in overall, the similarities tend to be more uniformly spread (Figure 4.2b,c). Remarkably, there are only a few changes in which enzymes retain a relatively high degree of sequence and functional similarity. For instance, the glycosyltransferase superfamily (CATH 1.50.10.20) exhibits a change of arabidiol synthase (EC 4.2.1.124) into thalianol synthase (EC 5.4.99.31) (circled in red in Figure 4.2a-c). This change involves different enzyme sequences from the terpenoid biosynthesis pathway of *Arabidopsis thaliana* that share high sequence identity (79%) and high reaction similarity (48% - bond change, 72% - reaction centre and 84% - structure similarity). They both act on (S)-2,3-epoxysqualene as the main substrate to synthesize a different product, which explains why the structure similarity is high.

Previous research presented how enzyme superfamilies evolve by a combination of chemistry-driven and substrate-binding-driven evolution (see 1.2 Evolution of enzyme function). In an attempt to analyse the chemical diversity of the domain superfamilies performing changes of function in isomerases, the functional similarity space was divided into four quadrants as depicted in Figure 4.2d. Each point represents a superfamily whose changes of isomerase function were averaged according to overall chemistry - as measured by bond change similarity - and structures of the reactants - in line with the similarity of the structures of substrates and products. Half of the superfamilies shared average similarities of reactants higher than 50% (top two quadrants), whereas only about one-fourth exhibited average similarities of overall chemistry higher than 50% (right two quadrants). Particularly, there are only three instances where the overall chemistry is similar but the structures of the reactants significantly diverge (bottom right quadrant), highlighting
Figure 4.2: Sequence and functional similarity of the 145 changes of isomerase function. The three scatterplots represent global sequence identity against overall reaction similarity as calculated using three measures (a) bond change (b) reaction centre and (c) structure similarity of substrates and products. Each point represents one change of enzyme function involving two sets of enzymes catalysing two distinct functions each (Furnham et al., 2012b). Average global sequence identities and standard deviations (error bars) are derived from all-against-all pairwise comparisons between sequences corresponding to one function and those corresponding to the second function. Encircled in red, the change EC 4.2.1.124 -> EC 5.4.99.31 (see main text). Pearson’s correlation coefficients (r) range from 0.35 to 0.41 and indicate weak but significant linear relationships (p-value < 0.001). (d) Distribution of bond change and structure similarities averaged by CATH superfamily.
that this is a rare event in the evolution of isomerase function.

### 4.3 An example - a family of SDRs acting on NDP-sugars from the UDP-galactose 4-epimerase superfamily

To explore one set of changes in more detail, eight changes of isomerase function involving a group of nine enzymes catalysing transformations between nucleoside diphosphate sugars (NDP-sugars) were studied. These metabolites are common in bacterial secondary metabolic pathways and they are necessary in molecular recognition and signalling processes (Singh et al., 2012). Several studies have revealed the structural, functional and mechanistic determinants of this group of evolutionarily-related enzymes. They are epimerases (EC 5), dehydratases (EC 4), decarboxylases (EC 4) and oxidoreductases (EC 1) belonging to the subfamily of short-chain dehydrogenases/reductases (SDR) acting on NDP-sugars (Figure 4.3a) (Eixelsberger et al., 2012; Frey & Hegeman, 2013; Hegeman et al., 2002; Kowatz et al., 2010). The changes in function involve two-domain enzymes comprising a catalytic NAD(P)-binding Rossmann-like domain (CATH 3.40.50.720) and a domain known as UDP-galactose 4-epimerase (CATH 3.90.25.10), which confers substrate specificity. The active site is located in the interdomain cavity where a conserved Tyr, Lys and Ser/Thr form a catalytic triad. Reactivity takes place on the C4, C5 and C6 atoms of the sugar substructure through a mechanism involving a transient oxidation intermediate mediated by NAD$^+$ (Tanner, 2008). The sequence data provide evidence that different catalytic amino acids are recruited to the active site in order to change the prevalent UDP-glucose 4-epimerase activity (EC 5.1.3.2) to other enzymatic activities. For instance, a base, Glu and an acid, Asp, are added to the catalytic triad in dTDP-glucose 4,6-dehydratase (EC 4.2.1.46) and GDP-mannose 4,6-dehydratase (EC 4.2.1.47) to perform the dehydration step which takes place in each of these overall reactions (Hegeman et al., 2002). Since the reactivity takes place in the attached sugar moiety, the nucleoside diphosphate substructure (noted as X in Figure 4.3a) is not disrupted during catalysis and remains conserved in all enzymatic activities of this superfamily.

FunTree catalogues 8 changes of isomerase function within this family of enzymes (Figure 4.3a). They all share the same domain composition and therefore changes in function result directly from changes in sequence, rather than domain architecture. The analysis of sequence and functional similarities revealed that this family is divergent, with members sharing sequence identities in the 20% to 40% range. Bond change similarities revealed the already observed bimodal distribution due to the EC classification definitions (Figure 4.3b). Similarities by reaction centre remain low - not higher than 50% (Figure 4.3c)
Figure 4.3: The evolution of SDRs acting on NDP-sugars. (a) Overview of the EC changes involving isomerases and domain composition of UDP-glucose 4-epimerases (EC 5.1.3.2). Biochemical reactions are represented in boxes. Black arrows inside boxes denote chemical transformations whereas coloured arrows linking boxes represent EC changes. EC numbers with an asterisk indicate reactions for which we found mechanistic evidence in MACiE (Holliday et al., 2012) or in literature searches. Changing substructures are highlighted in red whereas X corresponds to nucleoside diphosphate moieties (ADP, TDP, GDP, CDP, UDP) in which the base may change, but the ribose diphosphate (or sometimes the 2'-deoxy derivatives) is broadly conserved. Three scatterplots illustrating sequence and functional similarity for this superfamily (b) bond change, (c) reaction centre and (d) structure similarity of substrates and products as in Figure 4.2.
whereas overall, this set of functional changes tend to conserve structural similarity, due to the common binding of a conserved nucleoside diphosphate (Figure 4.3d).

Taken together, this overview of sequence and functional relationships may help to identify possible sequences catalysing orphan isomerase reactions. For instance, comprehensive literature and database searches confirmed that the enzymatic activity UDP-glucosamine 4-epimerase (EC 5.1.3.16) is an orphan EC number. In 1959, it was first experimentally determined in rat liver by Maley (Maley & Maley, 1959). The high functional similarity to the activities UDP-glucose 4-epimerase (EC 5.1.3.2), UDP-arabinose 4-epimerase (EC 5.1.3.5) and UDP-glucuronate 4-epimerase (EC 5.1.3.6) suggests that the sequence catalysing EC 5.1.3.16 may belong to the UDP-galactose 4-epimerase superfamily. Ultimately, experimental analysis will reveal whether candidate sequences actually perform this reaction.

4.4 Conclusion

Using isomerases as an example, this chapter explored how enzyme chemistry may change over time, as enzymes evolve to perform different enzyme reactions.

The observation is that isomerases are more likely to evolve new functions in different EC primary classes, rather than evolve to perform different isomerase reactions. This is unlike the other EC classes where more than two-thirds of the exchanges happen within the same EC class. In addition we note that exchanges between isomerases and lyases (EC 4) are prevalent.

Isomerases change their overall chemistry and conserve the structure of their substrates more often than conserving the chemistry and changing substrates. This is also unlike other types of enzymes and reflects the mechanisms of isomerases, which can often incorporate mechanistic components from different classes to provide a different overall outcome while conserving the substrate binding abilities.

The chaotic nature of the sequence and function relationship in superfamilies including isomerases is evidenced by the lack of correlation between sequence and functional similarity. Variations in sequence are always very large revealing that changes happened long ago, emphasizing that evolutionary studies need to be undertaken on a superfamily basis. Here we gave an example of how combining knowledge from the chemistry and evolution of enzymes acting on nucleoside diphosphate sugars may help to characterise related orphan activities.
Chapter 5

Characterising Complex Biochemical Reaction Data

Most research studies on enzymes assume a one-to-one relationship between biochemical reaction and Enzyme Commission (EC) number, the widely accepted classification scheme used to characterise enzyme activity. However, this is an oversimplified description and almost one-third of all known EC numbers are linked to more than one biochemical reaction. Whereas enzyme databases often try to resolve this complexity by defining generic, alternative and partial reactions, EC numbers are often linked to different types of reactions. This complexity adds a new dimension to our understanding of enzyme function and is relevant for improving the classification of enzymes and to study the change of enzyme function during evolution.

5.1 Complexity in the description of enzyme function

Enzymes are life’s catalysts that accelerate biochemical reactions up to the rates at which biological processes take place in living organisms. They play a central role in biology and have been thoroughly studied over the years. Since the 1960s, the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) has systematically encapsulated the functional information of enzymes into EC numbers. As discussed before, this is a hierarchical classification of enzymes based on multiple aspects of the overall chemistry of the biochemical reaction such as the chemical bonds that are broken or formed, cofactors being used and the nature of the substrates undergoing transformation (Tipton & Boyce, 2000). It is the global standard representation of molecular function for enzymes, and ultimately the genes, catalysing these transformations.
Whereas classical studies on enzyme catalysis highlighted the exquisite chemical specificity between enzyme and biochemical reaction (Fersht, 1999; Silverman, 2002), several studies have unearthed details of the additional ability of some enzymes to catalyse more than one biochemical reaction (Copley, 2003; O’Brien & Herschlag, 1999; Schulenburg & Miller, 2014). This phenomenon is widely known as enzyme promiscuity (Hult & Berglund, 2007); it has been described as being necessary for the evolution of enzyme function (Khersonsky & Tawfik, 2010); it has implications for biotechnology (Bornscheuer et al., 2012; Nobeli et al., 2009). Recently, various strategies to investigate the influence of chemistry in the evolution of promiscuous activities have been proposed (Khersonsky et al., 2011) together with approaches exploring evolutionary trade-offs between native and promiscuous activities (Garcia-Seisdedos et al., 2012). For such analyses, a transparent and accurate classification system to describe enzyme activity is particularly important.

The existing functional classification of enzymes has proved to be very powerful. It is manually curated and maintained by expert enzymologists, who use a controlled vocabulary and well-defined relationships in describing enzyme function (Friedberg, 2006) to convey the way biochemists think about reactions (McDonald & Tipton, 2014). It facilitates predefined comparisons between enzymes based on their chemistry and newly discovered enzymes are easily allocated in the different levels of its hierarchical classification. However, because of the diversity of chemical criteria used at different levels, the classification is not always coherent between EC classes (Kotera et al., 2004; Latino & Aires-de Sousa, 2006; Sacher et al., 2009). For instance, lyases (EC 4) are divided in subclasses depending on the type of chemical bond that is broken in the reaction whereas isomerases (EC 5) are divided based on the type of isomerisation. In addition, the EC classification is based on the overall catalysed reaction, which means that mechanistic steps and reaction intermediates are not considered. As a result, all enzymes carrying out the same overall reaction are generally assigned to the same EC number, even when they perform catalysis using different cofactors and mechanisms (O’Boyle et al., 2007).

For example, chloride peroxidase (EC 1.11.1.10) is used to describe three structurally distinct non-homologous enzymes, which change in their cofactor dependence in three different catalytic mechanisms and are deemed to have emerged from independent evolutionary origins (Holliday et al., 2011; Omelchenko et al., 2010). There are however exceptions to this rule, particularly in oxidoreductases (EC 1), where enzymes catalysing the same overall reaction using different cofactors are sometimes assigned different EC numbers. For instance, EC 1.1.1.32 and 1.1.1.33 represent two mevaldate reductases, both catalyse the conversion of (R)-mevalonate to mevaldate but respectively use NAD+ and NADP+ as a cofactor (Shearer et al., 2014). On the other hand, homoserine dehydrogenase (EC 1.1.1.3) has broader cofactor specificity and interconverts L-homoserine and
L-aspartate 4-semialdehyde using both NAD$^+$ and NADP$^+$ but with a slight preference for the first (Jacques et al., 2001).

Although reliable and rigorous, the manual process of naming and classifying each new enzyme is laborious and requires expert knowledge, therefore automatic approaches may help to accelerate this procedure. Similarly, the NC-IUBMB has also considered the current EC classification system to be a relic of the original attempts to develop a chemically sensible hierarchical classification. Ideas and methodologies envisioning a new system in which enzymes are assigned meaningless database identifiers have already been proposed (Tipton & Boyce, 2000) and automatic tools to search and compare enzyme reactions are useful to navigate through enzyme space and may help to improve future versions of the classification (Rahman et al., 2014).

There are also other limitations regarding the ability of the EC classification to accurately represent enzyme function (Babbitt, 2003). In fact, it had to be adapted after discovering that homologous enzymes annotated with the same EC number can manifest different levels of substrate specificity (Cornish-Bowden, 2014) (also known as substrate promiscuity or ambiguity). For instance, UDP-glucose 4-epimerases (EC 5.1.3.2) display different substrate specificities depending on the taxonomic lineage. Due to differences in the amino acid composition of the enzyme active sites, the bacterial epimerases only act upon UDP-glucose whereas the eukaryotic relatives additionally catalyse the transformation of UDP-N-acetylglucosamine (Daenzer et al., 2012). Similar trends of variation in substrate specificity are common for other isomerases (EC 5) (Gall et al., 2014) and enzymes from other EC classes (McDonald & Tipton, 2014). Even though this limitation has partially been addressed by introducing specificity information in the “Comments” section of several EC entries (Kotera et al., 2008) together with reaction data and structured tables in resources such as KEGG (Kanehisa et al., 2012) and BRENDA (Schomburg et al., 2013a), there is still a need to represent this phenomenon in a more computer-friendly format in order to obtain accurate comparisons between EC numbers.

Although enzyme function depends on the sequence and structure of the enzymes performing catalysis, the EC classification does not consider this information during the assignment process (Cornish-Bowden, 2014). However, scientific interest in groups of enzyme functions that are poorly classified by the EC (e.g. activities on polymeric biomolecules like sugars, proteins or DNA) led to the development of alternative schemes that consider homology as organisational principle. For instance, proteolytic enzymes are hydrolases (EC 3) and lyases (EC 4) exhibiting broad substrate specificity, classified using sequence and structure analyses in the MEROPS database (Rawlings et al., 2014). Likewise carbohydrate-active enzymes are classified in the CAZy resource using similar principles.
Currently, the EC classification is effectively used as the link between enzyme information (genes, sequence and structure) and chemistry data (biochemical reactions) in common resources like UniprotKB (The Uniprot Consortium, 2013). However there is already evidence suggesting that the correspondence between enzymes, EC numbers and biochemical reactions is not as simple as previously thought (Babbitt, 2003; Egelhofer et al., 2010). The relationship between enzyme and EC number is complex and rarely one-to-one (Holliday et al., 2011). Some enzymes are annotated with multiple EC numbers (multifunctional) (McDonald & Tipton, 2014) whereas some EC numbers are associated with many unrelated enzymes (Omelchenko et al., 2010). Early studies defined “average” sequence similarity thresholds for the accurate transfer of EC numbers to enzymes using homology (Rost, 2002; Tian & Skolnick, 2003; Todd et al., 2001) and subsequent efforts integrated other sources of information such as structural data (Laskowski et al., 2005b) and catalytic residue conservation (George et al., 2005) to help understanding this relationship. Although there are several studies that have deliberatively excluded multifunctional enzymes in order to avoid these complexities (des Jardins et al., 1997; Todd et al., 2001), some approaches aiming to predict biochemical reactions in metabolites have successfully handled reactions associated with more than one EC number (Mu et al., 2011). To some extent, KEGG circumvents the need for using EC numbers to link enzymes and biochemical reactions by directly connecting reactions to groups of orthologous enzymatic genes (Kotera et al., 2014). This association might considerably simplify the process of linking chemical and genomic information.

Driven by the observation of striking differences in the way biochemical reactions are represented using the EC classification in several databases (Altman et al., 2013), together with evidence from various studies excluding biochemical reactions associated with more than one EC number (Latino & Aires-de Sousa, 2009; Latino et al., 2008), here the relationship between EC number and biochemical reaction is explored. Although some reviews commented on certain aspects of EC number diversity (Bernard et al., 2014; Sorokina et al., 2014), to the author’s best knowledge, studies addressing this connection in a systematic manner are lacking. This relationship was first explored for a chemically diverse class of enzymes catalysing geometrical and structural rearrangements between isomers, the isomerases, and then this knowledge was used to develop an automatic approach to gain an overview of reaction diversity across the EC classification.
5.2 Characterising complexity

5.2.1 Overview

There are 5385 four-digit EC numbers in the 9th April 2014 release of the NC-IUBMB list, 4237 of them (79%) are associated with 6494 unique biochemical reactions bearing structural information in the 70.0+ release of KEGG database (Kanehisa et al., 2012), accessed using the KEGG website and Advanced Programming Interface (API) (Kawashima et al., 2003). The remaining 21% lack structural data. Although most EC numbers are linked to one biochemical reaction, almost a third are associated with more than one (Figure 5.1a). Comparatively, oxidoreductases (EC 1) exhibit the highest fraction of multiple reactions whereas isomerases (EC 5) the lowest (Figure 5.1b). Similarly, some unusual cases were identified where individual EC numbers are linked to over 20 biochemical reactions, with one extreme outlier, classified as an unspecific monooxygenase (EC 1.14.14.1) with up to 66 reactions (Figure 5.1c). In isomerases, the total number of EC numbers in the database is 245, for which 222 are associated with 298 biochemical reactions and 23 are not linked to any reaction. Among the EC numbers linked to isomerase reactions, 42 are associated with more than one reaction.

Figure 5.1: Survey of EC numbers associated with more than one biochemical reaction. (a) Overall distribution. White and gray slices indicate single and multi-reaction EC numbers, respectively. “R-group” represents EC numbers containing a Markush label in at least one reaction (see generic reactions in main text) (b) Distribution by EC class (c) Distribution of EC numbers according to the number of reactions.
5.2.2 Relationship between EC number and biochemical reaction in isomerases

In general, the intrinsic diversity in isomerase multi-reaction EC numbers was interpreted in terms of the chemical variability between the reactions linked to the same EC number. In the context of catalytic promiscuity, previous studies defined reactions to be different if they differ in the types of bond changes (formed and cleaved), the reaction mechanism or both (Kaltenbach & Tokuriki, 2014; Kazlauskas, 2005). The reactions associated with the 42 multi-reaction isomerase EC numbers were manually analysed on the basis of bond and stereochemistry changes and EC numbers were divided into three groups according to same, partial and different overall chemistry of the reaction (Figure 5.2). According to our observations, the first group was then further divided into two subgroups: different reactants and generic reaction. Since the EC number only describes the overall reaction, we do not include mechanisms in this analysis. Below is an explanation of each subgroup.

In the different reactants subgroup, reaction diversity arises due to the presence of different chemical substituents on a common structural scaffold. For example, the so-called “arginine racemase” (EC 5.1.1.9) describes the racemisation of arginine, lysine and ornithine. The three reactions involve a chiral inversion of the common Cα in the amino acid (Figure 5.2a).

Generic reactions are used to represent multiple reactions by means of the chemical composition of their reactants. They are represented using Markush labels (e.g. R-groups) (Brecher, 2008), which serve as chemical wildcards for other reactions. Almost one in five EC numbers are associated to at least one generic reaction, half of them refer to multi-reaction EC numbers and the other half represent single-reaction EC numbers (Figure 5.1a). Although the association between Markush labels from the generic reaction and the corresponding chemical substructures in exemplar reactions is direct for multi-reaction EC numbers, this correspondence in single-reaction EC numbers is challenging where comparisons with all the other EC numbers are required.

Multi-reaction EC numbers where at least one reaction is generic are the subject of this study. Generic relationships according to chemical composition are of two types. First, some cases resemble the characteristics of the different reactants subgroup but the various chemical substituents are collectively displayed in an additional generic reaction, which represents the rest of reactions. For instance, amino acid racemase (EC 5.1.1.10) is linked to five reactions. Four of them describe racemisations of glutamine, serine, ornithine and cysteine and the extra one represents all of them by encapsulating the diversity of the amino acid side chain into a R-group (Figure 5.2b). In some cases however, the generic
Figure 5.2: Examples of isomerase EC numbers associated with more than one biochemical reaction. (a) Arginine racemase (EC 5.1.1.9) is an isomerase acting on different reactants. The variability in chemical substituents is highlighted in green and the common scaffold in black. (b) Amino acid racemase (EC 5.1.1.10) is an example of generic reaction on the basis of R-group. Same colouring as in (a). (c) 2-acetolactate mutase (EC 5.4.99.3) is an example of generic reaction based on stereochemistry. The stereochemistry of C2 in acetolactate is represented as straight (undefined), up and down (defined) bonds and highlighted in green. (d) UDP-N-acetyl-D-glucosamine 2-epimerase (EC 5.1.3.14) belongs to partial reaction, (i) overall reaction - epimerisation of UDP-N-acetyl-α-D-glucosamine (green) and UDP-N-acetyl-α-D-mannosamine (blue), (ii) first partial reaction - hydrolysis and epimerisation of UDP-N-acetyl-α-D-glucosamine and (iii) second partial reaction - addition of UDP to N-acetyl-α-D-mannosamine. Intermediate compounds are highlighted in red. (e) Dichloromuconate cycloisomerase (EC 5.5.1.11) and 4-chlorobenzoyl-CoA dehalogenase (EC 3.8.1.7) catalyse different types of reactions. Shared bond changes are coloured in black, whereas different bond changes in green.
reaction is the common structural scaffold shared among all reactions. As a result, there is no R-group involved, and the reactants of the *generic* reaction are substructures of the reactants of the rest of reactions. For example, in Figure 5.2a the reactants in the epimerisation of L-ornithine are substructures of the reactants in the epimerisation of L-arginine, hence the former could also be a *generic* reaction of the latter. Although the latter *generic* relationship is evident in our manual analysis, in the process of developing an automatic method to assign EC numbers to reaction diversity groups (see 5.2.3 Automatic analysis) this was considered as an example of *different* reactants. Other isomerase EC numbers fall into this category such as chalcone isomerase (EC 5.5.1.6), which catalyses reversible cyclisation of chalcone into flavanone as common structural scaffold. In addition, it also performs the same reaction in hydroxy-substituted derivatives of chalcone and flavanone (Kimura *et al.*, 2001).

The second case of representation by *generic* reaction arises due to differences in the definition of stereochemistry between the *generic* reaction and rest of the reactions. Here, undefined stereochemistry (in the form of wiggly or non-stereo bond) characterises one of the chiral carbons in the *generic* reaction, whereas stereochemistry is defined for that atom in the rest of the reactions. Although a previous study reported data challenges due to the lack of stereochemical completeness in KEGG metabolites and reactions (Ott & Vriend, 2006), to some extent recent versions of the database have incorporated these recommendations to improve the handling of stereochemistry and related data inconsistencies. Taken together, the common existence of cases of defined and undefined stereochemistry in several EC numbers supported the formulation of this diversity group. For example, acetolactate mutase (EC 5.4.99.3) is associated with two reactions: the isomerisations of 2-acetolactate (*generic* reaction, undefined stereochemistry) and (S)-2-acetolactate (*specific* reaction, defined stereochemistry) (Figure 5.2c). As in *generic* reactions on the basis of R-group, cases of undefined stereochemistry in the form of wiggly bonds were detected in our automatic method, however the cases of non-stereo bonds were regarded as examples of *different* reactants.

It is a well known fact that there are enzymes releasing intermediate products of an *overall* reaction from the active site (McDonald & Tipton, 2014). Reactions leading to these intermediates are known as *partial* reactions. Similarly, an enzyme may subsequently catalyse two or more *partial* reactions with or without releasing any intermediates, these are considered as *consecutive* reactions. For example, in Figure 5.2d UDP-N-acetyl-D-glucosamine 2-epimerase (EC 5.1.3.14) catalyses the epimerisation of UDP-N-acetyl-α-D-glucosamine and UDP-N-acetyl-α-D-mannosamine (*overall* reaction). This transformation comprises two successive *partial* reactions in the mechanism - hence, they are *consecutive*. First, the UDP moiety is hydrolytically eliminated from the anomeric car-
Bon and epimerisation takes place at C2 (first partial reaction). Second, the UDP moiety is added to the anomeric carbon (second partial reaction). Combining these two consecutive reactions leads to the overall reaction. Whereas this example summarises this group in its simplest form, we also found three other alternatives of partial reactions linked to the same EC number:

- Two consecutive reactions and the overall reaction are all linked to the same EC number.
- Two consecutive reactions are linked to the same EC number, the overall reaction is linked to a different EC number.
- One consecutive reaction and the overall reaction are linked to the same EC number, the other consecutive reaction might be assigned to a different EC number or correspond to an uncatalysed reaction.

Previous studies have alternatively used the concept of “multi-step reaction” to refer to our definition of overall reaction composed of more than one partial reactions that occur consecutively (Kotera et al., 2004). However, the term step in a reaction usually implies one mechanistic step of the overall reaction. As mechanisms are not included in the EC classification, we preferred using the term partial reaction in order to avoid confusion.

Finally, EC numbers might also be linked to at least two different types of reactions. Dichloromuconate cycloisomerase (EC 5.5.1.11) catalyses two types: first, the isomerisation of 2,4-dichloro-cis,cis-muconate and 2,4-dichloro-2,5-dihydro-5-oxofuran-2-acetate and also, the conversion of 2,4-dichloro-cis,cis-muconate into trans-2-chlorodienelactone and chloride (Figure 5.2e) (Kuhm et al., 1990; Pieper & Stadler-Fritzsche, 1993). Although the two reactions share the cleavage of O–H and formation of C–O bonds, they differ in other bond changes, so they are considered to be different. However the product of the first isomerisation might eliminate chloride to yield trans-2-chlorodienelactone in an uncatalysed manner and therefore the second reaction would be the result of an isomerisation and successive elimination, which can also be interpreted as an example of partial reaction as described before. Other examples of EC numbers that can also be categorised under both different types of reaction and partial reaction involve sugar isomerisations such as those catalysed by D-arabinose isomerase (EC 5.3.1.3) and ribose-5-phosphate isomerase (EC 5.3.1.6) where the ring opening and closure might be uncatalysed. Perhaps a more definite example of different reaction types is 4-chlorobenzoyl-CoA dehalogenase (EC 3.8.1.7). This EC number involves the dehalogenation of 4-chlorobenzoyl-CoA into 4-hydroxybenzoyl-CoA and also the hydrolysis of the fluoro, bromo and iodo derivatives (Figure 5.2e).
Following our manual classification, 30 of the 42 multi-reaction isomerase EC numbers were solely assigned to one of the groups, whereas the diversity of the remaining 12 EC numbers was explained by more than one group. Overall, 57 group assignments were manually designated: 24 different reactants, 17 generic reactions (R-group and stereochemistry), 5 partial reactions and 11 different types of reactions. Among the EC numbers assigned to more than one group, we found 2-acetolactate mutase (EC 5.4.99.3) (Figure 5.2c). In addition to the transfer of a methyl group from C2 to C3 in (S)-2-acetolactate, this isomerase also catalyses the transfer of an ethyl group from C2 to C3 in (S)-2-aceto-2-hydroxybutanoate. This EC number could be assigned to both groups: generic reaction on the basis of stereochemistry and different reactants (Figure 5.3). Similarly, although dichloromuconate cycloisomerase (EC 5.5.1.11) is an example of different types of reactions (Figure 5.2e), a potentially uncatalysed elimination of chloride may also link these two reactions in a partial relationship.

2-Acetolactate mutase (EC 5.4.99.3)

*Generic reaction - stereochemistry*

2-acetolactate  \(\xrightarrow{\text{3-hydroxy-3-methyl-2-oxobutanoic acid}}\) 3-hydroxy-3-methyl-2-oxobutanoic acid

(S)-2-acetolactate  \(\xrightarrow{\text{3-hydroxy-3-methyl-2-oxobutanoic acid}}\) 3-hydroxy-3-methyl-2-oxobutanoic acid

(S)-2-aceto-2-hydroxybutanoate  \(\xrightarrow{(R)}\) (R)-3-hydroxy-3-methyl-2-oxopentanoate

*Different reactants*

Figure 5.3: 2-Acetolactate mutase (EC 5.4.99.3) is an example of EC number assigned to two groups of reaction diversity: different types of reaction and partial reactions.
5.2.3 Automatic analysis - extending diversity groups found in isomerases to the EC classification

The automatic extraction of chemical attributes from biochemical reactions such as bond changes and reaction centres is necessary to compare enzymes based on the chemistry of their catalysed reactions. In order to calculate chemical attributes EC-BLAST was used, a recently-developed algorithm to obtain accurate atom-atom mapping, extract bond changes and reaction centres and perform similarity searches between enzyme reactions (Rahman et al., 2014).

The strategy\(^1\) comprised a set of conditional statements combining bond change results from EC-BLAST, which allowed the detection of different types of reaction; comparisons of substrate and product structures and identification of R-groups and stereochemistry using Open Babel (O’Boyle et al., 2011) and in-house scripts, which helped to find generic and partial reactions (Figure 5.4). Cases of different reactants comprised the remaining multi-reaction EC numbers and were not detected by the conditions addressing the other diversity groups.

<table>
<thead>
<tr>
<th>Checks</th>
<th>Assignment</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>At least one reaction with R-group</td>
<td>Generic + R-group</td>
<td>Find chemical substituents for R-groups in the rest of reactions</td>
</tr>
<tr>
<td>At least one reaction with wiggly bonds</td>
<td>Generic + stereochemistry</td>
<td>Find stereochemistry for wiggly bond in the rest of reactions</td>
</tr>
<tr>
<td>Reactions have different bond changes (EC-BLAST)</td>
<td>Different types of reaction</td>
<td>Find overall and partial relationships</td>
</tr>
<tr>
<td>Explore order of reactants</td>
<td>Partial reaction</td>
<td></td>
</tr>
<tr>
<td>Previous checks found no hits</td>
<td>Different reactants</td>
<td></td>
</tr>
</tbody>
</table>

Figure 5.4: Workflow illustrating the automatic analysis of multi-reaction EC numbers.

\(^1\)The study of reaction diversity across the EC classification was done in collaboration with Handan Melike Dörentaş, a visiting student from the Department of Biological Sciences, Middle East Technical University, Ankara (Turkey). During her three months stay, Melike developed a method based on the 42 multi-reaction isomerase EC numbers to automatically label the type of diversity in any multi-reaction EC number (different reactants, generic reaction on the basis of R-group and stereochemistry, partial reaction and different types of reactions). My contribution to her project involved supervision.
The performance of the method was tested by assessing its ability to correctly identify the type of diversity in fifty randomly-selected multi-reaction EC numbers from the whole of the EC classification. The test dataset comprised 22 oxidoreductases (EC 1), 19 transferases (EC 2), 5 hydrolases (EC 3), 2 lyases (EC 4) and 2 ligases (EC 6), which were manually assigned to a reaction diversity group allowing performance to be evaluated. The selection of test multi-reaction EC numbers was carried out randomly, but it was assured that it covers the whole diversity space of the EC classification. True positives, true negatives, false positives and false negatives were calculated for each EC number in the test set and group of reaction diversity using manually-defined annotations as reference (Figure 5.5). Overall, the method successfully assigned the correct diversity group in 41 of the total of 50 test EC numbers. Almost all the assignments were true positives or true negatives, however nine remaining cases could not be correctly assigned due to data errors, detection problems and atom-atom mapping accuracy.

- EC 1.1.1.222 was assigned to different reaction types due to an atom-atom mapping error in R03337 and R03339. This issue was addressed in the present development version of EC-BLAST.

- EC 3.1.3.5 was assigned to different reaction types due to a data error, namely the different protonation state of the phosphate moiety in R02323 compared to the rest of reactions, leads EC-BLAST to detect a nonexistent formation of a O–H bond.
• EC 1.2.1.4 was not assigned to *generic* reaction + R-group due to a data error concerning protonation states. Whereas R00634 presents a carboxylate group, R00711 and R05099 consider the corresponding chemical species as carboxylic acid.

• EC 2.4.1.295 was not assigned to *generic* reaction + R-group due to difficulties in identifying R-groups. This is handled in development versions of the automatic method.

• EC 3.5.1.2 was not assigned to *generic* reaction + R-group due to a data error concerning protonation states. Whereas R06134 presents a carboxylate group, R01579 and R00256 consider the corresponding chemical species as carboxylic acid.

• EC 2.4.1.178 was not assigned to *generic* reaction + R-group due to similar difficulties in identifying R-groups as EC 2.4.1.295.

• EC 1.1.1.42 was not assigned to *partial* reaction due to difficulties in detecting *partial* reactions. Here, R00268 and R01899 are *partial* reactions of the overall R00267 such that R00267 = R00268 + R01899.

• EC 6.3.4.10 was not assigned to *partial* reaction due to similar difficulties in detecting *partial* reactions as in EC 1.1.1.42. Here, R01074 and R05145 are *partial* reactions of the overall R04582 such that R04582 = R01074 + R05145, considering C04763 = C06249 and C04727 = C06250.

• EC 1.1.1.100 was not considered in the test due to a data error. R04534 is unbalanced because a proton is missing on the right-hand side of the reaction.

5.2.4 Relationship between EC number and biochemical reaction in the EC classification

A schematic diagram illustrating the various groups of reaction diversity is shown in Figure 5.6a. There are 1277 multi-reaction EC numbers in the entire EC classification, 90% of them (1153) could be analysed using our method. The most common group was *different* reactants including almost half of the examples. *Different* reaction types followed with 29% and ultimately *partial* and *generic* reactions made up the rest (Figure 5.6b). The overall distribution was similar in oxidoreductases (EC 1), transferases (EC 2) and hydrolases (EC 3), which were correspondingly the EC classes involving the highest number of multi-reaction EC numbers (Figure 5.6c) and not surprisingly, also the EC classes with the largest number of EC numbers in the EC classification (McDonald *et al.*, 2009). Exceptionally, the most common diversity group in ligases (EC 6) is *different* reaction types, instead of *different* reactants. Also, the method did not identify any example of EC numbers involving *generic* reactions in lyases (EC 4) and ligases (EC 6).
5.3 Discussion

5.3.1 Overall

Although there is literature reported by the NC-IUBMB discussing specific cases of reaction diversity across the EC classification (McDonald & Tipton, 2014), the aim of this study was to systematically explore aspects of the chemical diversity in the description of enzyme function in a specific EC primary class - manually and automatically in the entire EC classification. In order to extract bond changes from biochemical reactions the EC-BLAST algorithm was used, which is based on chemical concepts, such as the principle of minimum chemical distance and chemical bond energies, in order to guide the atom-atom mapping and chemical matrices for similarity searches (Rahman et al., 2014). As suggested in a recent review by Chen et al. (2013), the incorporation of chemical knowledge adds accuracy to existing strategies to perform reaction comparison.
To what extent do the findings of this study overlap with those discovered in previous accounts on enzyme promiscuity? To some degree, the working definitions of substrate and product promiscuity (Hult & Berglund, 2007) somewhat resemble our diversity groups of different reactants and generic reactions. Likewise, catalytic promiscuity partly corresponds to different reaction types. However, whereas promiscuity definitions are genuinely attributed to enzymes in order to describe their ability to catalyse more than one biochemical reaction, our characterisation of reaction diversity applies to multi-reaction EC numbers, which adds an extra level of chemical variability to the existing definitions of enzyme function.

The surprising observation of this study is that almost one-third of the EC numbers involving more than one biochemical reaction have different reaction types, bearing key differences in catalysed bond changes. Whereas some of them also correspond to partial reactions, many are cases of catalytic promiscuity within the same EC number where the annotated enzyme catalyses two or more distinct reactions. Manual analysis revealed that most cases are similar to 4-chlorobenzoyl-CoA dehalogenase (EC 3.8.1.7) (Figure 5.2e) indicating that whereas some bond changes are shared, the rest individually characterise each of the different reactions.

The rationale behind why the NC-IUBMB and reaction databases have assigned multiple biochemical reactions to the same EC number is to some extent comprehensible. For instance, the product of some catalysed reactions sometimes undergoes a fast and uncatalysed reaction while still in the active site. These EC numbers comprise two reactions: one comprising only the catalysed reaction and another consisting of the catalysed+uncatalysed consecutive reactions. Whereas some enzymologists might preferably associate the EC number only with the catalysed reaction, the fact that the uncatalysed reaction takes place in the enzyme’s confinement supports the catalysed+uncatalysed interpretation.

However the complexity in the relationship between biochemical reaction and EC number goes beyond this study and cases of generic relationships are also common in single-reaction EC numbers (Figure 5.1a) and across different EC numbers. For example, as highlighted before, EC 5.1.1.10 was defined by the NC-IUBMB after the discovery of an enzyme that broadly catalyses racemisations of several amino acids (Lim et al., 1993). The biochemical reaction contains an R-group and it effectively represents reactions catalysed by specific amino acid racemases, which are also assigned different EC numbers, e.g. alanine (EC 5.1.1.1) and serine (EC 5.1.1.18). Although this and other examples (Kotera et al., 2014) were attempts to incorporate an enzyme property such as substrate specificity to guide the EC classification, this might lead in some cases to EC numbers being no
longer chemically independent from each other, which adds further complications to a classification based solely on the chemistry of the overall reaction.

5.3.2 Improving the EC classification

The ability of the NC-IUBMB to manually update the EC classification in the form of transferred and deleted entries when new enzyme data becomes available is necessary. For example, during the fifty years succeeding the creation of the EC entry for phosphoglycerate mutase in 1961 (EC 5.4.2.1), evidence supporting two distinct mechanisms concerning different usage of the cofactor 2,3-diphosphoglycerate by this enzyme accumulated in the literature (Foster et al., 2010). In 2013, the original EC number was transferred to EC 5.4.2.11 (cofactor-dependent) and EC 5.4.2.12 (cofactor-independent).

In addition, several expert recommendations concerning definition and handling of EC numbers in biological databases have already been suggested in different contexts. For example, Green and Karp advised about the problems associated with the assignment of partial EC numbers (those containing a dash, e.g. EC 5.1.1.-) to genes and proposed changes to the specification of these ambiguous identifiers (Green & Karp, 2005). Similarly, we suggest approaches to clarify multi-reaction EC numbers, which will hopefully help to improve the EC classification (McDonald & Tipton, 2014) and serve to guide standards for the reporting of enzyme data (Apweiler et al., 2010; Gardossi et al., 2010; Tipton et al., 2014) and existing initiatives for the assignment of enzyme function (Anton et al., 2013; Bastard et al., 2014; Gerlt et al., 2011).

A multi-reaction EC number belonging to the groups’ different reactants or generic reactions could either be combined into a single-reaction EC number (collective approach) or split into as many distinct EC numbers (specific approach). In the first place, diversity could be represented by R-group definitions, which would encapsulate chemical substituents at different positions in the reactants. When necessary, stereochemically-undefined bonds could also be employed to indicate the non-stereoselectivity of some biochemical reactions (Figure 5.7a). Secondly, the specific strategy arises when there are significant changes of substrate specificity between enzymes annotated with the same multi-reaction EC number. Instead of defining a generic reaction, it might be more sensible to re-define several EC numbers according to the distinct patterns of substrate specificity (Schomburg et al., 2014). However, although EC-BLAST provides a robust method to measure chemical differences between overall reactions in a continuous manner, defining the cut-offs required to designate separate EC numbers (for example, between different substrates) is a priori arbitrary and would need to be addressed explicitly.

A proposed modus operandi when dealing with different reaction types involves using the
Collective Specific
Arginine racemase (EC 5.1.1.9) 4-chlorobenzoyl-CoA dehalogenase (EC 3.8.1.7)

\[
\begin{align*}
\text{NH} & \quad \text{NH} \\
\text{O} & \quad \text{F} \\
\text{H} & \quad \text{2} \\
\text{O} & \quad \text{S} \\
\text{CoA} & \quad \text{O} \\
\text{HO} & \quad \text{HF} \\
\text{+} & \quad \text{+} \\
\end{align*}
\]

Figure 5.7: Examples of the collective and specific approaches. (a) The different reactants of arginine racemase (EC 5.1.1.9) are combined into a single-reaction EC number using R-group. (b) The two different types of reaction catalysed by 4-chlorobenzoyl-CoA dehalogenase (EC 3.8.1.7) are split and re-defined into two single-reaction EC numbers.

The specific approach to divide the multi-reaction EC number into multiple EC numbers, one for each different reaction (Egelhofer et al., 2010) (Figure 5.7b). Regarding partial reactions, we recommend to collectively reduce the multi-reaction EC number by combining all partial reactions with required enzyme catalysis into a single-reaction EC number, while setting uncatalysed reactions aside.

Both collective and specific approaches have several benefits. For instance, three main advantages characterise the collective approach. First, it is a compact way to arrange reaction information in a clear and structured manner. Second, it conveys how chemists and biochemists represent reactions in the literature, databases and patents (Geyer, 2013; Warr, 2011; Zass, 1990). Third, diversity can be captured using Markush labels such as R-groups (Brecher, 2008; Simmons, 1991), which would be subsequently described in associated files, tables or chemical libraries (Warr, 2014). Alternatively, diversity in the reactants could be encoded using recent developments in the description of chemical pat-
terns (Schomburg et al., 2013b). Also, the collective approach brings together reactions that are often evolutionarily-related. The precise definition of R-groups will also help previous studies that were limited in their ability to handle generic structures. Although some strategies did not explicitly define R-groups in their representation of biochemical reactions (Triviño & Pazos, 2010), several studies preprocessed oxidoreductase (EC 1) and hydrolase (EC 3) reactions by replacing every R-group by a hydrogen atom (Hu et al., 2010; Sacher et al., 2009) or methyl group (Mu et al., 2006) in order to calculate physicochemical and topological properties in atoms and bonds involved in reaction centres. Using more specific substitutions, R-groups were manually replaced by methyl, adenine, cytosine or other chemical moieties depending on the type of biochemical reaction (Latino & Aires-de Sousa, 2009; Latino et al., 2008). These studies suggest that having EC number-specific definitions of R-groups based on experimental evidence is a necessary step in order to implement the collective approach across the classification.

Whereas the collective approach relies on presenting a common structural scaffold and diversity encoded as chemical placeholders, the specific approach is divisive and explicitly distinguishes between reactions that are considered as chemically distinct. A clear advantage of the latter is when subtle differences between biochemical reactions are captured using different EC numbers, for instance, distinct bond changes. The description of enzyme function will then be more detailed and it will help to dissect some of the complexities in the relationship between enzyme sequence, structure and function (Holliday et al., 2011). The terms of application of the collective and specific approaches to combine or split multi-reaction EC numbers are summarised in the following recommendations:

- Reactions sharing the same overall chemistry (identical bond changes) should be combined into a single-reaction EC number (corresponding to groups: different reactants and generic reaction). The chemical diversity observed as different embodiments of a generic structure would be encapsulated using R-group definitions and stereochemically-undefined bonds in associated libraries and chemical patterns.

- If reactions have different overall chemistry (distinct bond changes), the EC number should be split in multiple single-reaction EC numbers (group: different types of reaction). Similarly, reactions catalysed by enzymes annotated with the same EC number that display distinct substrate specificities or cofactor dependencies should also be split in as many single-reaction EC numbers as patterns of specificity exist (groups: different reactants and generic reaction).

- Reactions sharing partial overall chemistry (several partial reactions integrate into an overall reaction) should be treated carefully. The partial reactions that take place in the active site of the enzyme should be combined into a single-reaction EC
number (group: partial reaction) with chemical diversity encapsulated in libraries as described before. Uncatalysed partial reactions should be considered separately. This systematic analysis is relevant for the functional annotation of sequenced genomes and by extension, it has implications for our ability to build and compare genome-scale metabolic reconstructions (Monk et al., 2014; Oberhardt et al., 2011; Saha et al., 2014). There is a direct correspondence between EC numbers and terms representing the molecular function of protein-coding genes in the Gene Ontology (GO) (Ashburner et al., 2000), which implicitly adopted EC numbers as part of their classification. This ontology is currently the widely-used standard for the automatic assignment of function to proteins and genes (Radivojac et al., 2013). We observed that multi-reaction EC numbers/GO terms are commonly transferred between similar enzymes during this process. Such a predicted assignment of function does not consider that enzymes annotated with the same multi-reaction EC number might have different reaction specificities in different species, which may lead to a general overestimation of the catalytic capabilities of organisms as predicted from their genomes.

5.4 Conclusion

This study adds an additional level of chemical complexity to our current description of enzyme function using EC numbers. Remarkably, almost a third of all known EC numbers are associated with more than one biochemical reaction. Existing approaches to characterise this diversity are ineffective, therefore this complexity was decomposed into four categories: different reactants, generic, partial and different types of reaction with the aid of computational methods to automatically compare biochemical reactions. Several recommendations to improve the characterisation were proposed, which will hopefully help to improve our understanding and description of biochemical reactions.
Chapter 6

Conclusions

The scope of this dissertation was to understand more about the chemistry and evolution of isomerase function (EC 5). Isomerases are life’s catalytic toolkit to interconvert isomers, molecules with the same molecular formula but different chemical structures. As one of the six classes in the EC system, isomerases populate the genome and contribute to the metabolism of all living organisms. They also serve in various applications for biotechnology and chemical synthesis. Like most enzymes, the overall chemistry of isomerases is captured in the EC system. Unlike for other EC primary classes (Holliday et al., 2014; Hu et al., 2010; Latino et al., 2008), an evaluation of the isomerase classification using a computational approach revealed the need for revision and improvement.

First, the EC system of isomerases needs simplification. The total of six subclasses can be summarised into two if isomerism is considered and the discovery of groups of similar overall reactions in complex sub-subclasses suggests that dissecting them into new sub-subclasses would help to resolve part of their complexity. Second, the prevalence of complex biochemical reaction data calls for more accurate description, especially multi-reaction EC numbers. A method to discover and classify complex biochemical reactions is currently under development. Reactions should be represented by the same EC number if the bond changes and the substrate’s reacting site are identical and split into different EC numbers if the bond changes differ, the reacting site is compromised or the substrate experiences an uncatalysed reaction occurring outside the enzyme’s active site. The best way to communicate these main findings and recommendations to the enzyme community, EC and reaction databases is currently under investigation.

The comprehensive study of the chemistry of isomerases helped to develop EC-BLAST, a tool to search and compare biochemical reactions (Rahman et al., 2014). A pipeline was produced to test the ability of the algorithm to detect changes of stereochemistry in isomerase reactions. It is based on trial and error and subsequently developed into a method which is routinely used today to check the chemical accuracy of the tool. EC-
BLAST provides a robust alternative to existing approaches to measure the functional similarity between proteins based on ontologies (Gabaldón & Koonin, 2013), although its range of application is limited to enzymes only.

During evolution, isomerase function often exchanges with other EC classes, especially lyases (EC 4). This is also unlike other EC classes where most exchanges occur within the same EC class (Furnham et al., 2012a). However, further analysis on whether this trend is a direct consequence of the EC classification of isomerases (Nath & Mitchell, 2012) or the result of chemical similarity between isomerases and other EC classes is needed. In addition isomerases conserve substrates and products more often than conserving bond changes. These observations suggest that isomerases might have originated from enzymes from other EC primary classes, which performed different overall chemistry in similar substrates and products. More analyses and experiments are needed to explore this hypothesis and to help to determine prevalent exchanges for other EC classes.

The EC classification of isomerase reactions only depends on aspects of the overall chemistry such as bond changes, functional groups and substrate specificity. In contrast to other EC primary classes such as oxidoreductases (EC 1), no mechanistic components e.g. cofactors guide the isomerase class. Therefore, the description of isomerase chemistry based on bond changes, reaction centres and substrates and products presented in this dissertation is enough to investigate coherence in the EC system.

However, the study of the evolution of enzyme function needs also to be informed by mechanistic data (Furnham et al., 2012a; Nath et al., 2014), which is not considered in this dissertation. For instance, although the analysis of bond changes and reaction centres demonstrated that racemase and epimerase reactions (EC 5.1) are similar in overall chemistry, these enzymes use different mechanisms such as proton or hydride transfers to produce enolates as catalytic intermediates. Twelve EC 5.1 mechanisms are available in MACiE and the four sub-subclasses are represented (Holliday et al., 2012). The means of producing and collapsing the intermediate are different between mechanisms. Some involve covalent catalysis using an organic cofactor such as pyridoxal 5'-phosphate (PLP) or NAD⁺ (Frey & Hegeman, 2013; Okazaki et al., 2009; Palani et al., 2013; Tanner, 2008), some stabilise the intermediate using a divalent metal cofactor like Mg²⁺, Zn²⁺, Ni²⁺ or Co²⁺ (Desguin et al., 2014; Petsko et al., 1993) and some do not employ any cofactor but only a pair of catalytic amino acids acting as conjugate acid and base, respectively. For instance, the bacterial glutamate racemase (EC 5.1.1.3) first uses a cysteine to deprotonate the substrate L-glutamate, which leads to a planar enolate intermediate. Then, the intermediate collapses by deprotonating a second cysteine and the product D-glutamate is generated. Taken together, mechanisms add an extra layer of functional information
to evolutionary analyses and increasing the coverage of resources like MACiE using the enzymology literature seems necessary. Alternatively, in order to inform an overall chemistry representation with mechanistic components, Gasteiger and coworkers proposed a physicochemical description of the reaction centre (Hu et al., 2010; Rose & Gasteiger, 1994). However, this approach does not include fundamental aspects of enzyme mechanisms such as catalytic amino acids and cofactors, which are considered in other methods that compare enzyme mechanisms directly (Almonacid et al., 2010).

From the chemistry point of view, this study depends on the quality of reaction data available in databases (Ott & Vriend, 2006). To some extent the manual curation efforts tried to resolve some discrepancies, however many reactions were not balanced therefore atom-atom mapping becomes impossible. Similarly, stereochemistry assignments are sometimes not valid. Whereas multiple strategies to correct unbalanced reactions (Chen et al., 2013; Kraut et al., 2013; Shaw et al., 2012) and to reconcile biochemical reactions across databases (Bernard et al., 2014) have been recently presented, novel improvements of the algorithms and further data curation and integration are essential (Kumar et al., 2012). In addition, the quality of the manual curation performed in this dissertation depends on the author’s ability to interpret reactions, as well as the experimental information available in the primary literature. Overall, the analysis relied only upon the reaction equation and the performance of EC-BLAST to compute accurate atom-atom mappings.

Our study of the evolution of isomerase function relies on the quality of functional annotations in the reviewed section of UniprotKB and PDB (Berman et al., 2013; The Uniprot Consortium, 2013). Recent studies exploring biases in functional annotations discovered that the reviewed section of UniprotKB presents the lowest levels of misannotation compared to other resources (Furnham et al., 2009; Schnoes et al., 2009, 2013). As a result, several strategies were developed to automatically detect misannotations based on sequence and genomic context correlations (Hsiao et al., 2010) and history of changes in functional annotations (Silveira et al., 2014).

How useful is it to have a functional classification of isomerases based on chemical attributes? In general, classification brings order to chaos and helps to extract knowledge from structured data. In the context of this study, classification using EC-BLAST is useful to navigate and compare overall reactions. Although the method is automatic, quantitative and consistent, the best way to classify isomerase function depends on the purpose of the classification and requires the development of criteria. Although the EC classification which is based on overall chemistry is the current standard for reporting isomerase function, several studies have recently shown that other aspects such as substrate
substructures not directly involved in the reacting moiety also contribute to the catalytic efficiency of enzymes (Khersonsky et al., 2011) and affect how enzyme function evolves (Furnham et al., 2012a; Martinez Cuesta et al., 2014). A recent study explored how changing substrates by removing functional groups that are distantly located from the reacting chemical bonds can considerably reduce the catalytic activity of enzymes (Barellier et al., 2014). Therefore combining bond changes and reaction centres with structural information about the substrates and products and mechanisms is needed to capture the essence of enzyme chemistry in a functional classification. This might help to connect overall reactions to mechanisms and to study the evolution and design of enzyme function.

In the 1980s, the divergence of the amino acid sequence and three-dimensional structure of related proteins was first shown to be monotonic (Chothia & Lesk, 1986), which led to the foundations of homology-based protein structure prediction. Soon after, researchers turned into sequence-based database searching to discover that sequence similarity is also correlated to functional similarity so functions of unknown proteins can be transferred from those of their homologues up to certain extent (Friedberg, 2006). Exceptionally, a single mutation can drastically change function (Schmidt, 2003; Terao et al., 2006) or unrelated proteins might also perform the same function (Omelchenko et al., 2010). In fact, current algorithms for protein function prediction from sequence take this relationship for granted using machine learning principles (Radivojac et al., 2013). However the shape of the association is still unknown. In this dissertation, the relationship between sequence and functional similarity in superfamilies containing isomerases was shown to be remarkably nonlinear, yet monotonic. Other approaches to obtain similarity between enzymes based on the three-dimensional structures or catalytic sites need to be explored in future studies.

The analysis of the evolution of isomerase function is based on exploring the evolution of separate domains. However many enzymes are multidomain and change their domain composition and function during evolution (Bashton & Chothia, 2007). In addition, enzyme function is assigned on a whole-sequence basis without associating specific functions to the composite domains (Lopez & Pazos, 2009). Therefore cataloguing the functional evolution of each individual domain is a complex process, which can lead to multiple different evolutionary pathways. Further research on domain-based assignment of enzyme function and more experimental studies exploring how enzyme function changes with multidomain architecture would complement and broaden this dissertation.

In order to understand more about the relationship between the chemistry and evolution of enzyme function, further research is crucial. This dissertation is isomerase-centric therefore other EC classes need to be investigated. Although the methods are useful
to explore and improve the EC classification, understanding the relationships between sequence, structure, function and mechanism requires more work. It now seems even more obvious that future efforts should continue revolving around the question of how enzymes evolve new function. In order to address this fundamental topic, more experimental data is required. Choosing a family or a metabolic pathway to study in detail is not easy and depends on what is already known and the feasibility of conducting experiments. Each family or pathway is complex and different, hence in order to address the problems of finding candidate sequences for orphan enzymes or searching for potential enzyme activities of proteins with unknown function, each family or pathway will have to be considered separately. To be able to continue exploring the origins of biochemistry and further developing enzyme design, these issues need to be resolved in the near future. The field of high-throughput determination of protein function could not be better positioned to help to address the challenges of enzyme research today.
Bibliography


Greenacre, M. 2007 *Correspondence Analysis in Practice*. Taylor and Francis, 2nd edn.


Nguyen, T. T., Fedorov, A. A., Williams, L., Fedorov, E. V., Li, Y., Xu, C., Almo, S. C. & Rauschel, F. M. 2009 The mechanism of the reaction catalyzed by uronate isomerase illustrates how an isomerase may have evolved from a hydrolase within the amidohydrolase superfamily. *Biochemistry, 48*(37), 8879–90. (doi: 10.1021/bi901046x)


Oksanen, J. 2011 Multivariate Analysis of Ecological Communities in R : vegan tutorial.


Paradis, E. 2012 *Analysis of Phylogenetics and Evolution with R*. Springer.


Penny, D. & Hendy, M. D. 1985 The Use of Tree Comparison Metrics. *Syst. Biol.*, **34**(1), 75–82. (doi: 10.1093/sysbio/34.1.75)


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White, D. & Gramacy, R. B. 2012 maptree: Mapping, pruning, and graphing tree models.


