

# How Nature deals with stereoisomers

Victor S Lamzin, Zbigniew Dauter and Keith S Wilson

European Molecular Biology Laboratory, Hamburg, Germany  
and University of York, York, UK

All natural proteins are composed of L-amino acids and are inherently chiral. The properties of both L- and chemically synthesized D-amino acids are identical except in optically asymmetric interactions. Structural studies of D-L racemic mixtures of crystallographic interest are discussed. The review also gives some recent examples of stereospecificity: how L-proteins deal with L- or D-substrates and how enzymes can function as racemases. Two particular examples of stereoselectivity are then discussed.

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## Introduction

“Chirality is a property of any geometrical figure if its image in a plane mirror, ideally realised, cannot be brought to coincide with itself” (Lord Kelvin, 1893) [1].

The word ‘chiral’ is derived from the Greek word ‘cheir’, meaning hand. It is well known from geometric principles that any three points always lie in a plane. If the number of points is increased to four, they no longer need to lie in a plane and therefore become candidates to serve as a chiral geometric structure. In organic substances, by definition containing carbon atoms, a tetrahedral  $sp_3$  hybridized carbon with four different substituents will always be a chiral centre. A substrate carbon atom is not chiral if two out of four of its substituent groups are equivalent. It may however be ‘prochiral’ if a subsequent enzymatic reaction, resulting in a product, affects one of these two groups [2]. The same is true of a substrate which has an  $sp_2$  carbon which becomes  $sp_3$  in the product [2]. Therefore a protein–ligand interaction is stereospecific if either the substrate or product has a chiral centre.

A widely used nomenclature defines a chiral carbon atom as being *R* (derived from the Latin *rectus* for right) or *S* (*sinister* for left). The carbon is viewed from the direction opposite to the substituent atom of lowest atomic mass. If the atomic mass of the remaining three substituents decreases in a clockwise direction, the configuration of the chiral carbon is *R*, otherwise it is *S*. Another nomenclature uses *D* for right- and *L* for left-handed chiral centres (Fig. 1). Both nomenclatures are used in this article. However, *R/S* enantiomers are defined on the basis of a chiral centre, whereas *D/L* were originally based on optical rotation.

Chirality, as an inherent property of organic compounds which are products of biological processes, was first described by Louis Pasteur [3]. A further breakthrough

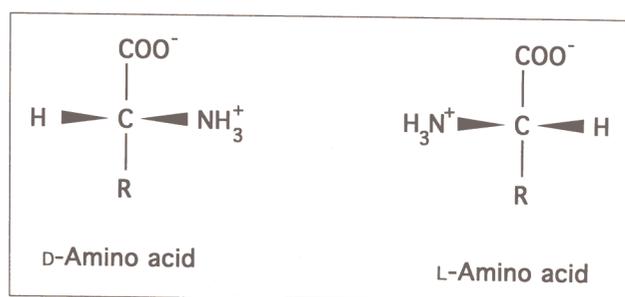


Fig. 1. The D- and L-configuration of amino acids, the building blocks of proteins. Only the L-enantiomer is found in natural proteins and this is the source of chirality in biological molecules.

was made by Emil Fischer [4], who suggested that biological macromolecules were composed of chiral L-amino acids and D-sugars. Natural substrates or ligands can have either an L- or a D-configuration for those centres where the reaction takes place.

The cornerstone of protein–ligand chiral recognition is multi-point attachment theory [5] which states that at least three of the four substituents on the chiral carbon atom should interact with the protein. Indeed, if only two of the groups interact, interchange of the other two may not affect the protein–ligand interaction.

Every major category of biological molecules is chiral, including proteins, nucleic acids, polysaccharides, lipids and steroids. If polymers, they are built up of units possessing chiral centres and the resulting three-dimensional fold is chiral. Indeed, any polymer chain folded into a three-dimensional structure will generally adopt one of two mirror images: for proteins where the individual units are chiral, only one of the two images is energetically feasible. Only one of these mirror images is synthesized by living organisms, as such syntheses all depend on the action of proteins composed of L-amino acids. In this sense, all enzyme reactions are in

## Abbreviations

3D—three-dimensional; FDH—formate dehydrogenase; MR—molecular replacement; NAD—nicotinamide adenine dinucleotide.

principle stereoselective. It is clear that natural enzymes with L-amino acids must in their interactions with other chiral macromolecules such as nucleic acids and polysaccharides show stereoselectivity. As this applies to all such reactions they will not be discussed here. We concentrate consequently on recent studies involving synthesis and properties of D-amino acid proteins, and of interactions of enzymes with smaller substrates where stereoselectivity is important for academic or commercial reasons.

### Proteins composed of D-amino acids

Natural proteins include only L-amino acids. Some exceptions do exist: the cell walls of Gram-negative bacteria contain a D-alanine, and a number of small oligopeptides made as antibiotic agents by a variety of microorganisms contain some D-amino acids. However, recent advances in methods of protein synthetic chemistry [6] mean that it is now feasible to synthesize complete D-amino enantiomers of at least small proteins.

One of the first examples of this was a study of HIV-1 retroviral proteinases [7]. The structure of the naturally occurring L-enzyme, the HIV-1 proteinase with 99 amino acids, had been determined earlier [8]. The D-enantiomer was synthesized by chemical methods and its properties compared with those of the L-enzyme [9]. Circular dichroic spectra showed equal but opposite optical rotations for the two enantiomers. These were also shown to be equally active on hexapeptide substrate analogues, the D-enzyme only being active on D-peptides and the L-enzyme on L-peptides. Study of a set of chiral pseudo-peptide inhibitors showed that they only inhibited enzymes of the same chirality. In contrast, an achiral inhibitor, Evans Blue, was an effective inhibitor of both enantiomers of the enzyme. These results are in full agreement with a true mirror imaging of the two tertiary folds and a consequent mirroring of their activity on chiral substrates. D-enzymes are indeed active on the inverse enantiomers of the natural substrates.

X-ray crystallography is the most widely used technique for revealing the complete 3D structure of all the atoms in a molecule. It can indeed provide information on the optical enantiomer present in the crystal. A central problem in X-ray crystallography is the lack of a lens system for focusing X-rays — the so-called phase problem. This means that phases must be determined by one of a number of methods. The solution is generally trivial and overdetermined for small molecules, but often challenging for macromolecules (for a review, see [10]). For each of the thousands of reflections with measured amplitude, the phase must be determined before the electron density can be computed. For non-centrosymmetric structures, such as proteins, the phase can take any value in the range 0–360°. However, for centrosymmetric structures, the phases are restricted to one of two values, 0 or 180°. In this case the phase problem is greatly simplified and the mean

accuracy of the phases at the end of the analysis is improved, giving potentially improved electron-density maps. Cocrystallization of enantiomers as a means of simplifying the phase problem was suggested by Mackay [11]. Crystallization of racemic mixtures of chiral molecules may lead to a centrosymmetric crystal lattice. A caveat is that crystallization can lead alternatively to separation of the enantiomers into different non-centrosymmetric crystals; indeed, this was exactly the method of purification of racemates pioneered by Pasteur [3].

Three structures have been reported for molecules containing racemic mixtures of D- and L-enantiomers in centrosymmetric space groups, two for oligopeptides and one for the protein rubredoxin. In each case the D-polypeptide was produced by chemical synthesis. The first structure was that of the small peptide Leu-enkephalin [12•].

The second was the decapeptide trichogin A IV [13••], which belongs to the family of peptaibols, linear peptides of fungal origin containing a large number of amino isobutyric acid residues. Trichogin modifies the lipid structure of membranes and is of considerable biological interest. Data were recorded on crystals of the naturally occurring L-protein but only extended to ~2.0 Å resolution, creating a challenging problem for phase determination and structure solution. The racemic mixture led to crystals containing two molecules (165 non-hydrogen atoms) in the asymmetric unit, but for which data could be recorded to 0.9 Å. It is not clear why the racemic crystals diffracted so much better. A straightforward application of direct methods using SHELXS [14] solved the phase problem without prior knowledge of the molecular structure.

The third example of racemic protein crystals is of the rubredoxin originally from *Desulfovibrio desulfuricans* [15]. The molecule contains a single chain of 45 amino acids folded around an FeS<sub>4</sub> cluster, and contains no typical secondary structural elements. The structure of the natural L-protein had already been determined at 1.5 Å resolution [16]. A racemic mixture was crystallized in a centrosymmetric space group, with one molecule in the asymmetric unit. Data on the racemic crystals were only recorded to the lower resolution of 2.0 Å; it is not clear that this was the absolute limit of the diffraction. The limited resolution precluded a solution by challenging modern techniques such as direct or Patterson methods [17]. Instead, the structure was solved by molecular replacement (MR) using the L-protein as a model. The centrosymmetry resulted in the phases from the initial MR being correct for 80% of the most intense four fifths of the reflections. The authors report the maps as being of very high quality for this resolution [15].

These results confirm that D-proteins/polypeptides share the physicochemical properties of the natural L-enantiomers but possess inverse structures. Apart from this inversion, they possess no special difference in properties that might shed light on the selection of L-proteins

during evolution; but more detailed studies are required to clarify this conclusion. It is not clear how general the use of racemic mixtures in crystallographic studies will become. This depends on how quickly techniques in protein synthesis develop relative to those useful for solving the phase problem for natural L-proteins. It will be some time before a D-enantiomer *Escherichia coli* bacterium is created, thereby allowing modern cloning techniques to be applied to D-enzymes.

### How dehydrogenases process mirror-related substrates

As stated above, enzymes are intrinsically asymmetric and are selective for the chirality of the substrate. For example, natural proteinases have well defined binding sites which recognize only L-polypeptides and do not bind to the D-enantiomers [18]. We wish to address the problem of the processing of mirror-image substrates. Clearly, a protein will in general possess an active-site geometry which selects one of the two isomers and does not bind or process the other. Few examples of structures of such systems exist. Here, we review results for the nicotinamide adenine dinucleotide (NAD)-dependent dehydrogenases.

The NAD-dependent dehydrogenases compose perhaps the most widely studied family of proteins after the serine proteinases. The first 3D structures had already been discussed in terms of their catalytic and evolutionary relationships over 20 years ago [19]. The field has remained of major interest and functional and structural studies are continually emerging. Investigation of NAD-dependent dehydrogenases is further justified by their biotechnological potential, as they are promising candidates for coenzyme regeneration systems in the asymmetric synthesis of high-value-added products such as amino acids and steroids [20].

The original series of structures comprised L-dehydrogenases (alcohol, lactate and malate). Each of these are dimeric or tetrameric, with two domains in each monomer. They all contain a six-stranded parallel  $\beta$ -pleated sheet domain which binds the dinucleotide at the C-terminal end. The six-stranded sheets show no significant primary structural similarity, but nevertheless share the same topology, comprising two elements of what is today referred to as the 'Rossmann fold' [21]. A common sequence and structural fingerprint have been identified for binding NAD, suggesting a common ancestor for all of the coenzyme binding domains [22]. All these enzymes are specific for L-2-hydroxy acids: the active site has a basic asymmetry which is clearly adapted for L-substrates.

Another family of dehydrogenases, the so-called D-specific 2-hydroxy acid dehydrogenases, exhibit exactly inverse specificity [23]. These proteins catalyze reactions with D-hydroxy acids and carry out both hydride and proton transfer. NAD-dependent formate dehydrogenase (FDH), which metabolizes the only non-chiral

substrate in this series, the formate anion, is by sequence homology a member of this family [24], as was first pointed out to us in 1992 by John Holbrook (personal communication). The reaction catalyzed by FDH is not stereochemically specific and comprises hydride transfer only. FDH was the first member of the family for which a 3D structure was determined [25]. The tertiary fold and quaternary structure differ substantially from those of L-specific NAD-dependent dehydrogenases, and FDH shares marginal sequence homology with these proteins only in the nucleotide-binding region [26]. Structures have subsequently been determined for two other D-specific dehydrogenases [27,28].

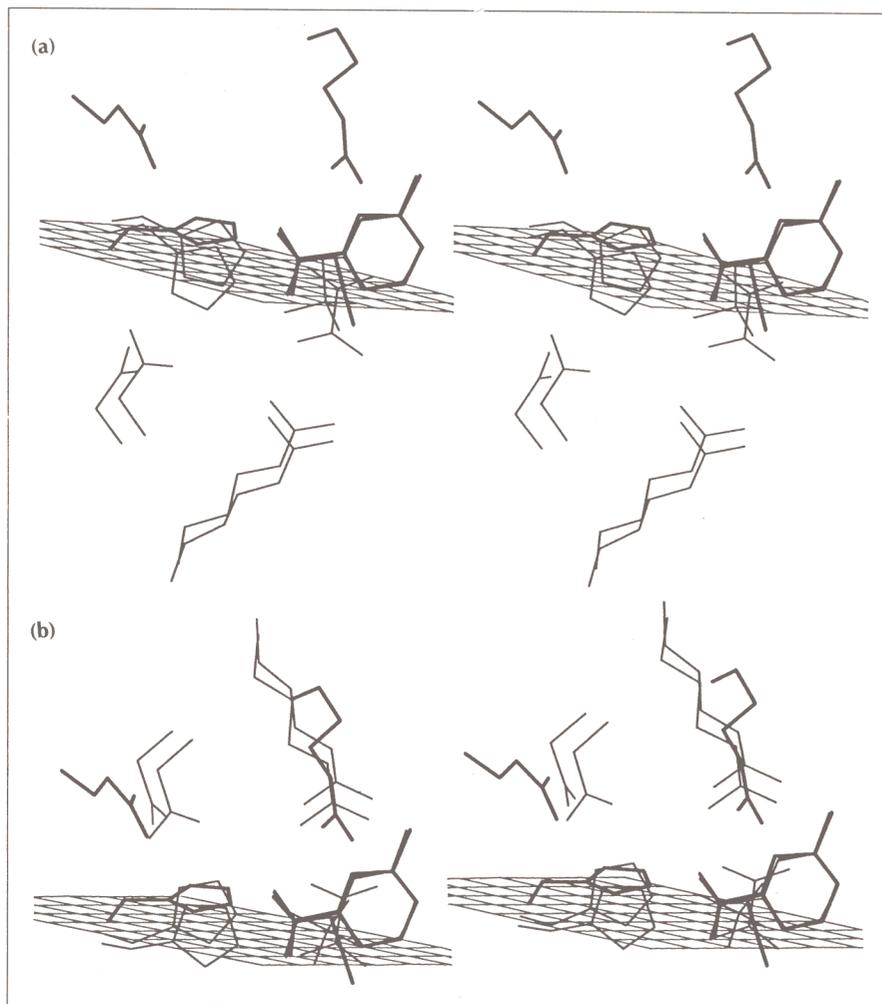
The catalytic mechanism of the L-specific enzymes is well understood [19,29]. The catalytic site involves an arginine residue which anchors the carboxylate group of the substrate and a histidine residue which provides the proton transfer and interacts with the 2-hydroxyl group. The histidine is paired with an aspartate to form a charge relay system first observed in the active centre of chymotrypsin [30]. Inspection of the structure of FDH [25] also revealed an arginine residue crucial for substrate binding and a histidine in close proximity. As Nature herself cannot produce mirror images of the complete enzyme, the only possibility is that the active site is a mirror of that found in the L-dehydrogenase family, if a similar mechanism is involved.

The FDH structure was compared with those of the L-specific enzymes [31]. The key atoms of the three catalytic residues (arginine, histidine and aspartate) of the L-specific enzymes were inverted through a mirror and superimposed on the equivalent atoms in FDH (Fig. 2). The similarity of the inverted structures was striking for enzymes with no sequence homology. Moreover, the mirror plane passes through the catalytic point in NAD, the C4N atom. This was confirmed by the subsequent determination of the structures of the two D-specific dehydrogenases, which indeed have active sites homologous to that of FDH. Thus, in the D- and L-specific dehydrogenases, inversion of stereospecificity is achieved by mirror inversion of the arrangement of the three catalytic residues.

### Interconversion of racemates

We now turn to enzymes that catalyze the reversible interconversion of R- and S-enantiomers through abstraction of protons from the  $\alpha$ -carbons of carboxylic acids. This family includes *inter alia* mandelate racemase, muconate lactonizing enzyme and galactonate dehydratase. These enzymes generate and stabilize enolic intermediates from either or both sides of the active site, and the processing of the intermediate to product is determined by the particular architecture of the active site. Thus the chiral active site of the enzyme must recognize both enantiomers of the substrate equally well.

Mandelate racemase has been most widely studied from the structural point of view [32,33]. The forward and



**Fig. 2.** Stereodiagrams of the mirror image relationship between the active-site residues of L-specific lactate and malate dehydrogenases and of formate dehydrogenase, homologous to the D-specific enzymes redrawn from that presented in [31]. The nicotinamide moiety in the three structures are superimposed. (a) The superposition of the original structures. (b) The superposition after the L-enzymes have been reflected through the mirror plane.

reverse reactions have equal  $k_{cat}/K_m$  values, according to the Haldane relationship. The reaction basically involves the abstraction of a proton adjacent to the carboxyl group, the so-called  $\alpha$  proton, by general base catalysis. Deuterium/hydrogen exchange studies and overshoot experiments were interpreted as follows. S-Mandelate has its  $\alpha$ -hydrogen removed by a polyprotic base ( $\epsilon$ -group of a lysine residue) whereas removal of the R-mandelate hydrogen requires a monoprotic base (imidazole of a histidine residue). This was confirmed by the X-ray crystal structure, which showed Lys166 and His297 to be the two active-site bases responsible for abstracting the  $\alpha$ -protons. The couplet of His297 paired with an aspartic residue serves as a general base catalyst.

The catalysis proceeds through increasing acidity of the  $\alpha$ -hydrogen by involving a glutamate residue serving as a general acid and making an H-bond with the carboxylate group of the substrate; a bivalent magnesium cation plays a crucial role. Lys166 is the S-specific base and abstracts the proton from S-mandelate. His297 is the R-specific base and abstracts the proton from R-mandelate. The immediate product of proton abstraction from either enantiomer is an enolic intermediate, and protonation of this intermediate by the conjugate acid, which can

be either Lys166 or His297, leads to the product enantiomer. Site-directed mutagenesis and X-ray studies of mutants have confirmed this. Mutations were introduced that constrained the enzyme to catalyzing the reaction in one direction only.

The racemization catalyzed by mandelate racemase requires both lysine and histidine. The  $\beta$ -elimination catalyzed by the homologous muconate lactonizing enzyme has a lysine in the same position as Lys166 but does not have a histidine equivalent to His297. The  $\beta$ -elimination catalyzed by galactonate dehydratase has a residue equivalent to His297, but no residue equivalent to Lys166. It has been proposed that the lysine and histidine general bases found in the active site of mandelate racemase evolved independently to generate enzymes that catalyze three different reactions associated with this family.

An extended protein family comprises proteins that behave similarly to mandelate racemase [34]; these include alanine,  $\epsilon$ -caprolactam, proline and diaminopimelate racemases. The reactions catalyzed can proceed by either a one-base (after abstraction of an  $\alpha$ -proton the intermediate carbanion is protonated back randomly, giving either substrate or product) or two-base mechanism (both abstraction and protonation are affected by two

enantiomer-specific catalysts located on both sides of the chiral carbon/planar carbonium intermediate), depending on the enzyme. Hence the enzymes of this group have differing features of specificity. For example, proline racemase exists in two, *R*- or *S*- enantiomer-specific forms. Each of the forms is able to process only one of the proline isomers. This is a direct result of the presence of two bases on either side of the substrate and the fact that these two bases may, under some conditions, differ in their protonation state. In effect, the *R*-form of the protein contains the *R*-specific base which is not protonated and can abstract the  $\alpha$ -proton from the *R*-substrate, and the *S*-specific base acts as the conjugate acid in the second step of the reaction. In the *S*-state of the enzyme the situation is reversed.

An intrinsic property of enzymes such as racemases is that the catalysis proceeds through a planar carbanion intermediate. The carbon atom in the intermediate is prochiral and, having accepted a proton from either side of the plane, is transformed into one of the two possible chiralities.

### Lipases

Lipases (triacylglycerol hydrolases) catalyze hydrolysis and transesterification of not only glycerol and cholesterol but also a broad range of synthetic esters which can serve as precursors to pharmaceuticals, agrochemicals and other targets. Lipases are particularly useful in such applications as they combine two opposing properties: acceptance of a broad range of substrates and retention of high enantioselectivity. As stable and inexpensive enzymes, lipases are often used by organic chemists for synthesis of enantiomerically pure compounds [35,36]. The structures of a number of lipases have been determined in recent years. Their mechanism is based on a catalytic triad (serine, histidine and glutamate/aspartate) similar to that found in the subtilisin and trypsin-like serine proteinases [37].

Cyglér and colleagues [38\*\*] have presented results on the structural basis of enantiomer preference in lipases. The sequences of all lipases for which structural information is available contain a consensus motif, Gly-X-Ser-X-Gly, encompassing the active-site serine. The chiral centre of the substrate has four substituents: hydrogen, hydroxyl group, a large and a medium organic group. Lipases can process both enantiomers but have clear preference for one over the other. They resolve relatively poorly secondary alcohols with substituents of similar size but discriminate more efficiently if the two groups differ substantially in size.

An example of chiral selection in lipases is a study of a fungal enzyme from *Rhizomucor miehei* [39]. The enzyme was incubated with a racemic mixture of a transition-state analogue, phosphonate ester. Crystals were grown and subsequent X-ray analysis showed that only a single stereoisomer was covalently bound to the active-site serine.

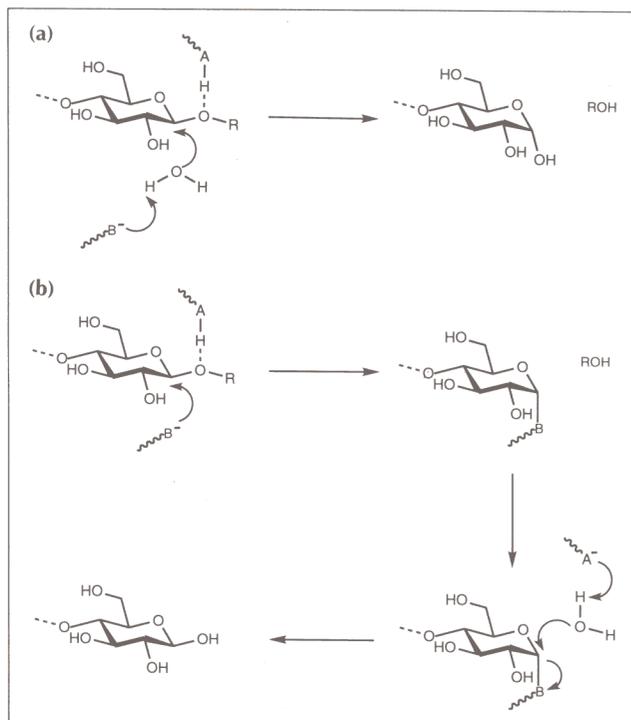
A full understanding of the basis of the chiral preference is not really available. Crystallographic studies suggest that binding of the poorer enantiomeric substrate is constrained by the steric properties of the active site, with the large substituent on the alcohol requiring a change in conformation of the catalytic histidine. This causes weakening of an H-bond to the substrate OH group in the complex and results in differential reactivity for the two enantiomers. In lipases, the chirality of the active site gives a preference for one of the two enantiomers without completely discriminating against the other. The degree of selectivity varies between different lipases, depending on the active-site architecture.

### Glycosyl hydrolases

We now turn to a different problem: the hydrolysis of the glycosidic bond between adjacent sugars. Glycosyl hydrolases are of interest in the present context because they all split the same type of linkage using mechanisms that involve at least one carboxylic group on the protein and often two. The glycosidic linkage can vary considerably in type, with the configuration at the anomeric carbon being  $\alpha$  or  $\beta$ , and the other carbon in the adjacent sugar being usually at the 3, 4 or 6 position. The monosaccharides on each side of the linkage may vary within a whole host of different sugars, carrying a range of substituents. Glycosyl hydrolases are generally highly specific for the type of glycosidic link and the constituent sugars.

The first enzyme structure to be determined was that of a glycosyl hydrolase, lysozyme [40]. In recent years, interest in this field has become intense. The structures are now classified into 45 families based on known sequences [41,42], and the number is continuing to expand. Three-dimensional structures are now known for representatives of 22 of these families and the structural work has been recently reviewed [43\*].

The glycosyl hydrolases exhibit two basic catalytic mechanisms, giving inversion or retention of configuration at the anomeric carbon (Fig. 3) (Non-enzymatic hydrolysis in solution yields a mixture of both anomers: enzymes selectively produce but one). Both mechanisms proceed by general acid catalysis, and require a proton donor and a base. The donor is always a protonated carboxylate group on the protein, positioned in the same orientation with respect to the glycosidic oxygen of the substrate in both mechanisms. The base is usually a negatively charged carboxylate residue, but a tyrosine has been implicated in some structures [44]. The position of the base varies between the two mechanisms [45\*]. In retaining enzymes, it lies  $\sim 5.5 \text{ \AA}$  from the donor and very close to the anomeric centre. It directly stabilizes the oxocarbenium ion intermediate, perhaps with a covalent link. In contrast, in inverting enzymes, the basic residue is located further from the anomeric centre and is indeed separated from it by a water molecule, which causes the inversion of anomeric configuration. The base lies on average  $10 \text{ \AA}$  from the donor.



**Fig. 3.** A schematic representation of the two alternative mechanisms of glycosyl hydrolases. (a) Inversion. (b) Retention of the anomeric configuration.

Nature has evolved families of glycosyl hydrolases with totally different 3D folds to split the glycosidic bond. Variation in positioning of the base relative to the donor and substrate gives simple and elegant variation in the chirality of the anomeric carbon. In addition, the stereochemistry of the catalytic and substrate sites gives a whole spectrum of substrate specificities for different sugars. Totally different 3D folds can produce somewhat similar catalytic sites: the important factor is to stabilize the appropriate relative position of a small number of key atoms.

## Conclusion

All of the macromolecules, and indeed most of the small ones, of living organisms are chiral. Hence essentially all of their interactions show stereospecificity. Enantiomeric pairs that show absolutely equivalent physicochemical properties in a symmetric environment can behave remarkably differently in the biological world, depending on their hand.

One application in this field is the chemical synthesis of D-proteins to catalyze reactions involving substrates and products of the opposite hand to those that occur naturally. D-proteins find a further application in allowing the growth of centrosymmetric racemic crystals. How extensive such studies will become remains to be seen.

The use of natural L-proteins in stereoselective syntheses such as those mentioned for lipases is already substantial.

With the increasing insistence of the drug and food additive regulatory commissions on the presence of only the active enantiomer without its inactive enantiomer in commercial products, the importance of understanding the structural basis of such selectivity and how to change and exploit it through site-directed mutagenesis is clear.

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VS Lamzin, European Molecular Biology Laboratory (EMBL), c/o DESY, Notkestrasse 85, D-22603 Hamburg, Germany.  
E-mail: victor@embl-hamburg.de

Z Dauter, KS Wilson, European Molecular Biology Laboratory (EMBL), c/o DESY, Notkestrasse 85, D-22603 Hamburg, Germany and Department of Chemistry, University of York, Heslington, York YO1 5DD, UK.

Z Dauter e-mail: dauter@yorvic.york.ac.uk

K Wilson e-mail: keith@yorvic.york.ac.uk