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DOI

[10.1002/cctc.201901589](https://doi.org/10.1002/cctc.201901589)

Publication date

2019

Document Version

Final published version

Published in

ChemCatChem

Citation (APA)

Marsden, S. R., Mestrom, L., McMillan, D. G. G., & Hanefeld, U. (2019). Thermodynamically and Kinetically Controlled Reactions in Biocatalysis – from Concepts to Perspectives. *ChemCatChem*, 12(2), 426-437. <https://doi.org/10.1002/cctc.201901589>

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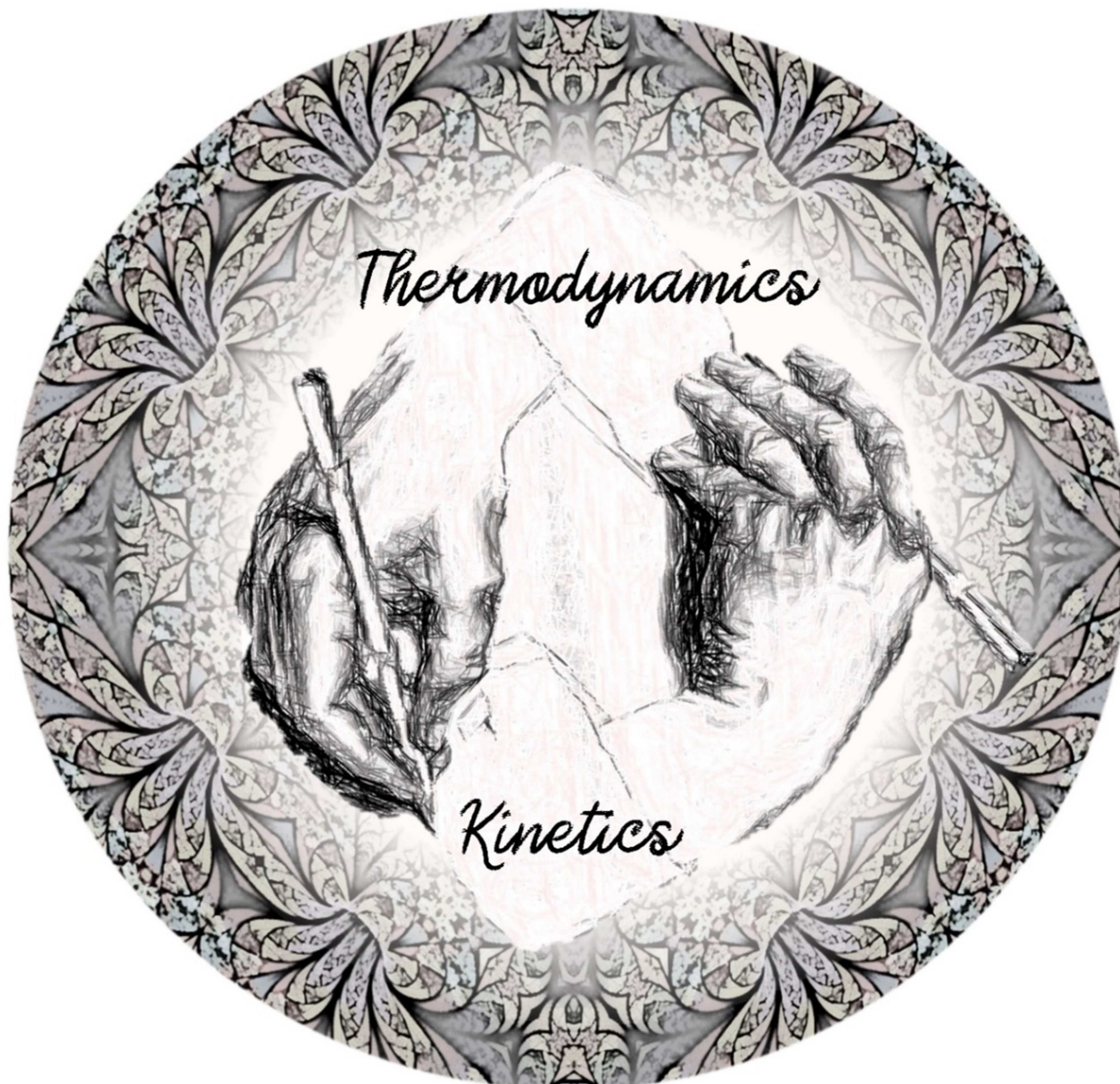
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Thermodynamically and Kinetically Controlled Reactions in Biocatalysis – from Concepts to Perspectives

Stefan R. Marsden,^[a] Luuk Mestrom,^[a] Duncan G. G. McMillan,^[a] and Ulf Hanefeld^{*[a]}

Dedicated to *Dick Janssen* on the occasion of his retirement.



The enzymatic synthesis of esters and peptides is unfavoured in aqueous solvent systems due to competing hydrolysis. This can be overcome by using energy rich substrate analogues: elimination of a good leaving group temporarily establishes more favourable equilibrium conditions, allowing for (nearly) complete conversion. While kinetically controlled syntheses of esters and peptides in water are common knowledge in biocatalysis textbooks, the prevalence of kinetic control is less well known for other enzyme classes. Here, the general concepts of thermodynamic and kinetic control are illustrated at the example of the well-studied synthesis of β -lactam antibiotics and are shown to similarly also apply to other

enzyme classes. Notably, the enzymatic synthesis of diastereomers shows the same characteristic energy profile as that of Diels-Alder reactions. This allows for the selective synthesis of different diastereomers under either thermodynamically or kinetically controlled conditions. Prospects and pitfalls of this notion are discussed at the example of the thermodynamic epimerisation of hydroxysteroids and recent examples of kinetically controlled aldol reactions. Kinetic reaction control can therefore not only be used to increase conversions towards a single product, but also to selectively afford different diastereomers. This review highlights the prevalence of both concepts within the field of biocatalysis.

Introduction

Catalysis plays a central role for the transition towards more sustainable production processes of chemicals and a circular economy. Biocatalytic approaches are particularly promising due to the typically high chemo- regio- and enantioselectivity of enzymes, which often renders the use of extensive protecting group strategies unnecessary.^[1] This feature allows for the design of more direct synthesis routes, effectively decreasing the number of reaction steps that are required to reach the final product.^[2] Biocatalytic approaches therefore often allow for substantial improvements in the *E-factor* (which is defined as the amount of waste generated per kilogram of product)^[3] by omitting waste intensive purification steps of reaction intermediates in one-pot cascade reactions^[4] and avoiding the use of stoichiometric reagents with low atom economy. However, some reactions are inherently limited to low conversions by their thermodynamic equilibrium, which inevitably also leads to a large *E-factor* and threatens the economic viability of the process, even when enzymes are used as catalysts.

Practical solutions to equilibrium limited reactions typically include the addition of one of the substrates in excess, the *in-situ* removal of (side-) products by evaporation or phase separation, or follow up reactions of (side-) products to drive the overall reaction.^[5] The enzymatic synthesis of esters or peptides in water is such an example, where equilibrium conditions strongly favour the corresponding alcohol/amine and acid starting materials, for which conversions of less than 1% are commonly observed.^[6] However, energy rich substrate analogues can be used to increase the change in free energy through the elimination of a good leaving group, which then

allows for (near) complete conversion of the starting materials under kinetically controlled conditions (Figure 1). In this way, high transient yields can be obtained, but the elimination of an additional leaving group constitutes a drawback from an environmental perspective as it lowers the overall atom economy.

In the first part of this review, the general concept and characteristics of a kinetically controlled reaction for increased product yields are highlighted at the example of the well-investigated synthesis of β -lactam antibiotics. Based on this, parallels are drawn to other enzyme classes which similarly catalyse kinetically controlled reactions, but have not yet been studied in as much detail. Furthermore, the actual impact of using substrate analogues on the atom economy is analysed for representative reactions for each enzyme class.

Historically, thermodynamic and kinetic control were first described for Diels-Alder reactions, which can proceed through multiple possible transition states of varying activation energies. Due to the intrinsic energy difference between diastereomers, thermodynamics favour the formation of one particular diastereomer under equilibrium conditions according to Equation (1):

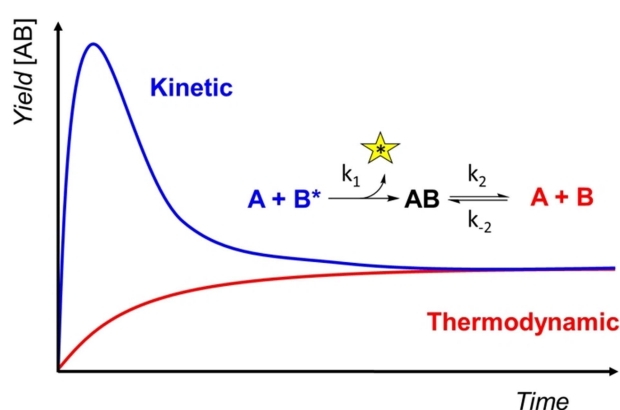




Figure 1. Typical progress curves of product concentrations (AB) in reactions under kinetic control (blue) or thermodynamic control (red). The substrate analogue B* contains a high energy leaving group (*) and its elimination temporarily establishes more favourable equilibrium conditions. Upon dissipation of the initial driving force, the equilibrium between AB and A + B becomes predominant and the product concentration then converges towards that of the corresponding thermodynamically controlled reaction.

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$$K_{eq} = \exp\left(\frac{-\Delta\Delta G}{RT}\right) \quad (1)$$

where $\Delta\Delta G$ is the free energy difference between the diastereomers, R the universal gas constant, T the temperature and K_{eq} the corresponding equilibrium constant. In cases where the desired molecule does not constitute the lowest energy product, its synthesis then essentially needs to be kinetically controlled by close control of the reaction temperature and time.^[7] Strikingly, while thermodynamically and kinetically controlled Diels-Alder type reactions have continued to be the subject of many scientific studies until today,^[8] no similarly detailed conceptual studies have been reported for the enzyme catalysed synthesis of diastereomers.

In the second part of our review, similarities between the energy diagrams of Diels-Alder reactions and the enzymatic synthesis of diastereomers are therefore highlighted in the context of thermodynamic and kinetic control. Examples from recent literature indicate, that while elements of kinetic control were previously observed during the enzymatic synthesis of diastereomers, they rarely are identified as such and therefore remain largely unexplored. With the continued emergence of thermostable enzymes, biocatalytic transformations are increasingly carried out at elevated temperatures; conditions which are known to favour the formation of thermodynamic products. Insufficient awareness of the possible competition between the target kinetic molecule and the corresponding thermodynamic product could therefore constitute a formidable pitfall during

enzyme discovery and evolution for applications in asymmetric synthesis.

Kinetic Control for increased Product Yields

Concepts and Prerequisites

Reactions which release only a small amount of Gibbs free energy are intrinsically limited to low conversions, while other reactions can also become limited by the process conditions. For instance, water is considered to be an environmentally more benign alternative to organic solvent systems, yet, competing hydrolysis of many target molecules such as esters and peptides can render their synthesis unfavourable under such conditions.^[6,9] The elimination of a good leaving group from a substrate analogue offers a possible solution, by releasing a larger amount of free energy which temporarily allows for increased conversions (Figure 1).

This effect is transient, as the target molecule remains catalytically interconnected with the thermodynamically less favourable reaction. Upon dissipation of the driving force that was released by the elimination of the leaving group, the reverse reaction becomes dominant again and the product concentration ultimately converges back towards the equilibrium concentration of the corresponding initial substrates. In order to successfully increase conversions by means of kinetically controlled conditions, two separate criteria must therefore be met: Firstly, the substrate analogue must be chosen in a way



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continued this work firstly in Germany (Friedrich Schiller Univ.), then as an JSPS international fellow in Japan (The Univ. of Tokyo). He was awarded the Prestigious Rutherford Discovery Fellowship in 2016, and has since taken up his present position at TU Delft.

Ulf Hanefeld was born in 1966 in Köln, Germany, and grew up in then (West) Berlin and London. In 1993 he received his PhD from the Georg-August-Universität zu Göttingen, having performed the research both with Prof. H. Laatsch (Göttingen) and Prof. H. G. Floss (Seattle). After postdoctoral years with Prof. C. W. Rees (Imperial College London), Prof. J. Staunton (Cambridge) and Prof. J. J. Heijnen and Dr. A. J. J. Straathof (TU Delft), he received a fellowship from the Royal Netherlands Academy of Arts and Sciences (KNAW). He rose through the ranks at the Technische Universiteit Delft and his research in Delft focuses on enzymes, their immobilisation and application in organic synthesis.

that elimination of the leaving group releases sufficient additional free energy to increase the equilibrium constant to an extent, which then allows for (near) complete conversion. Notably, the elimination of a gaseous leaving group (e.g. by decarboxylation) and its release from the reaction mixture can help with driving the reaction, but is not a prerequisite for successful kinetic control. An overview of free energy changes and the corresponding equilibrium constants for representative thermodynamically and kinetically controlled reactions is shown in Table 1 at the end of this section. Secondly, the rate constant for the conversion of the substrate analogue must be sufficiently larger than that of the corresponding reverse reaction, as they are competing throughout the entire course of reaction ($k_1 \gg k_2$, Figure 1).

The ratio of (k_1/k_2) is an important characteristic of kinetically controlled systems, as it determines the maximum achievable yield.^[10] For acyl transferases, the ratio of (k_1/k_2) is termed the synthesis to hydrolysis ratio and is commonly used to compare the efficiency of different enzymes in catalysing a specific kinetically controlled reaction. The rates of conversion for both the substrate analogue (k_1) and the product (k_2) typically follow Michaelis-Menten type kinetics, where k_{cat} and K_M vary for each structural analogue.^[6,9,11]

The individual choice of substrate analogue therefore influences the outcome of the reaction in two ways: first, the release of additional free energy determines the maximum extent of conversion that is possible by *thermodynamics*.^[12] Second, its individual rate constant of conversion then determines the ratio of (k_1/k_2), and thereby the maximum transient product yield that is possible by *kinetics*.^[9] The ratio of (k_1/k_2) is therefore an important property of the particular catalytic system and should be reported for all kinetically controlled reactions to allow for comparison. In cases where both rate constants are within the same order of magnitude, addition of several equivalents of the substrate analogue can become necessary to achieve the desired level of conversion.^[9]

It is worth mentioning that the substrate analogue, which contains the leaving group, is termed the 'donor substrate' in kinetically controlled reactions. This feature must not be

confused with its role during the reaction, where it can either act as nucleophile or electrophile.

In the following, the application of kinetic control to increase product yields under thermodynamically unfavourable conditions is described for five different enzyme classes to illustrate the widespread utility of this concept within biocatalysis. However, this overview is by no means comprehensive, and many enzymatic reactions involving activated substrates are being continually developed. One such example is the recently described enzymatic Friedel-Crafts acylation with activated acyl donors, which is mechanistically reversible and is therefore also expected to be kinetically controlled.^[13]

Amidohydrolases

Enzymatic amide bond formation is one of the key steps in the synthesis of many semi-synthetic β -lactam antibiotics (e.g. amoxicillin or ampicillin) and is being industrially applied annually on a multi-ton scale.^[5b] Notably, the production of semi-synthetic β -lactam antibiotics involves both the thermodynamically controlled hydrolysis and subsequent kinetically controlled synthesis of an amide bond, where both reactions are catalysed by the same enzyme (Scheme 1). This reaction requires a high regioselectivity to prevent the undesired hydrolysis of the energy rich β -lactam, which makes enzymes the preferred catalysts for this transformation.

Penicillin G (PenG) or penicillin V (PenV) are produced as precursors by fermentation, and hydrolysis of the amide bond affords 6-aminopenicillanic acid (6-APA) as the key building block for β -lactam antibiotics. Conversion of 6-APA with the appropriate acyl donor under kinetically controlled conditions subsequently affords the different members of the β -lactam antibiotic family.^[14]

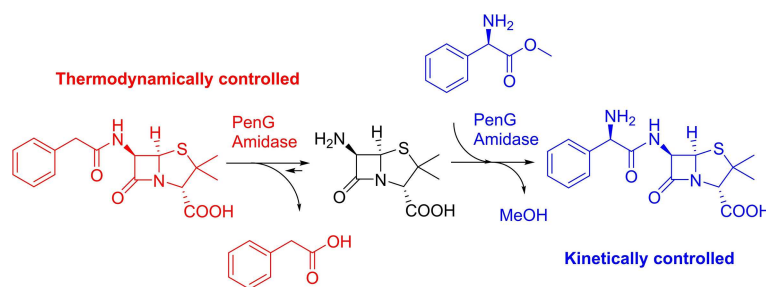
Due to its industrial relevance, the synthesis of amide bonds is, to our knowledge, the most well studied example of kinetically controlled reactions in biocatalysis, and extensive reviews have previously been published elsewhere.^[10,15]

For thermodynamically controlled reactions, the maximum product yield is determined by the corresponding equilibrium

Table 1. Overview of (transient) equilibrium constants for representative reactions under kinetic control. Over time, the product concentration converges towards the equilibrium constant of the corresponding thermodynamically controlled reaction. The elimination of water during esterification, glycosylation and phosphorylation reactions leads to a non-quantitative atom economy also for most of the corresponding thermodynamically controlled reactions. The difference in atom economy between thermodynamically and kinetically controlled approaches largely depends on the molecular weight of the reactants and can be as little as 3.5% percentage points in the case of ampicillin synthesis.

Donor	Acceptor	Product	Atom economy [%]	$\Delta_r G^{\circ [a]}$ [kJ/mol]	$K_{eq} [b]$
Hydroxypyruvate	Glycolaldehyde	Erythrulose	70.6	-264.5	2.2×10^{46}
Glycolaldehyde	Glycolaldehyde	Erythrulose	100	-4.0	5.0
UDP-Glucose	Glucose	Trehalose	45.9	-13.2	207
Glucose	Glucose	Trehalose	95	11.7	9.0×10^{-3}
Vinyl acetate	Benzyl alcohol	Benzyl acetate	77.3	-43.2	3.8×10^7
Acetic acid	Benzyl alcohol	Benzyl acetate	89.3	26.6	2.1×10^{-5}
ATP	D-glyceraldehyde	D-G3P	28.8	-23.3	1.2×10^4
Phosphate	D-glyceraldehyde	D-G3P	91.5	3.1	0.28
D-Phenylglycine methylester	6-APA	Ampicillin	91.6	n.a.	n.a.
D-Phenylglycine	6-APA	Ampicillin	95.1	n.a.	n.a.

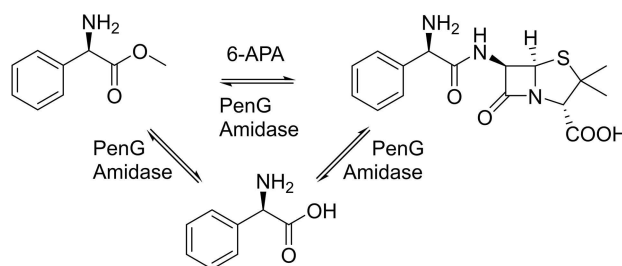
[a] Calculated for pH 7.0 and 0.1 M ionic strength with eQuilibrator,^[34] [b] Calculated from the change in Gibbs Free energy.



Scheme 1. Thermodynamically controlled hydrolysis of PenG to 6-APA, followed by a kinetically controlled condensation with D-phenylglycine methylester to afford the broadband antibiotic ampicillin.

constant and therefore strongly depends on process parameters such as temperature, pH, ionic strength, water activity, presence of organic co-solvents and the molar ratio of substrates used.^[10] For instance, elevated reaction temperatures decrease the equilibrium constant for exergonic reactions ($\Delta G < 0$), whereas endergonic reactions ($\Delta G > 0$) become more favourable at higher temperatures according to Equation (1). The pH is an important process parameter, as it influences both the change in free energy and the reaction kinetics. In many reactions, the protonation state of functional groups majorly influences their reactivity and this is also the case for the synthesis of amides. Carboxylate groups have a pK_a in the range of 3, whereas amino groups have a pK_a in the range of 8. Since the reaction occurs between the two uncharged species, a pH value equal to the arithmetic mean of their pK_a values then constitutes a compromise in terms of reactivity. The presence of organic co-solvents can decrease the K_a values of carboxylic acids by up to several orders of magnitude. This improves the relative amount of reactive substrates by rendering their corresponding pK_a values more similar.^[16]

In contrast, the maximum achievable product yield of the corresponding kinetically controlled reaction is determined by the synthesis to hydrolysis ratio (k_1/k_2). This is an intrinsic property of the enzyme, which is derived from its affinity (K_M) and catalytic rate constants (k_{cat}) towards the different substrates that are present in the reaction mixture. In the kinetically controlled synthesis of β -lactam antibiotics, the desired acyl transfer reaction is in constant competition with both the enzyme catalysed and non-catalytic hydrolysis of the acyl donor and of the product. This increased formation of by-products makes subsequent downstream processing more difficult and constitutes the major drawback to the kinetically controlled approach (Scheme 2). Hydrolysis of the β -lactam product can be reduced by its extraction into the organic phase in biphasic systems,^[17] or by its precipitation.^[18] Finally, the synthesis to hydrolysis ratio is also influenced by the method of enzyme immobilisation, where hydrophilic resins have given better results than hydrophobic resins.^[17,19]

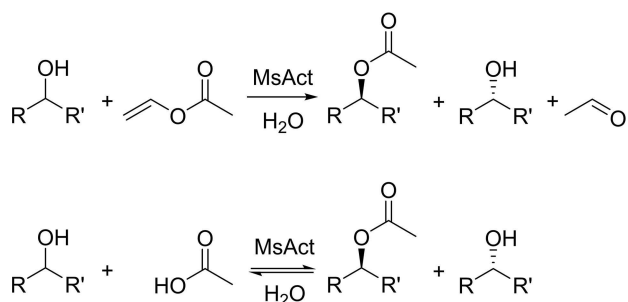


Scheme 2. Competing hydrolysis of both the acyl donor and product to phenylglycine. The formation of salt waste renders downstream processing more difficult and constitutes the major drawback to the kinetically controlled approach.^[17]

Acyl Transferases

Acyl transferases catalyse the synthesis of esters and amides and are frequently applied in (dynamic) kinetic resolutions for the production of enantiopure amines and alcohols. To render these processes economically feasible, kinetically controlled conditions with an appropriate acyl donor are used to achieve the required levels of conversion. Following the rule of Kazlauskas,^[20] one of the two enantiomers gets preferentially converted during kinetic resolutions and its separation from the unreacted enantiomer then becomes straightforward. The performance of an enzyme to catalyse kinetic resolutions is reflected in the *E ratio*, which can be calculated from the individual rate constants of conversion for each enantiomer.

Notably, the reactivity of the acyl donor must be carefully tuned to match the conditions (Scheme 3). On the one hand, a high reactivity of the substrate analogue (k_1) is required to establish an economically acceptable synthesis to hydrolysis ratio (k_1/k_2), whereas an excessively high reactivity results in the non-catalytic conversion of both enantiomers and reduces the overall performance of the kinetic resolution. It is noteworthy that esters can also be enzymatically synthesised in dry organic solvents or under neat conditions, which offers an alternative solution to the unfavourable thermodynamic equilibrium of ester synthesis *in aqua*.^[21] Nonetheless, enzymes generally require a minimum water activity to display their full potential and undesired reverse hydrolysis also remains a challenge in non-aqueous solvent systems.^[22] However, enzymatic reactions in dry organic solvents and the influence of water activity lie



Scheme 3. Representative acyl transferase catalysed kinetic resolution of a chiral alcohol *in aqua* using vinyl acetate as an acyl donor. Elimination of vinyl alcohol drives the reaction towards higher transient conversions and tautomerism to acetaldehyde renders the by-product non-nucleophilic. Competing hydrolysis of the product ester requires the reaction to be kinetically controlled (top).^[24] The corresponding thermodynamically controlled reaction with acetic acid is limited to low conversions, making it economically unfeasible (bottom).^[9]

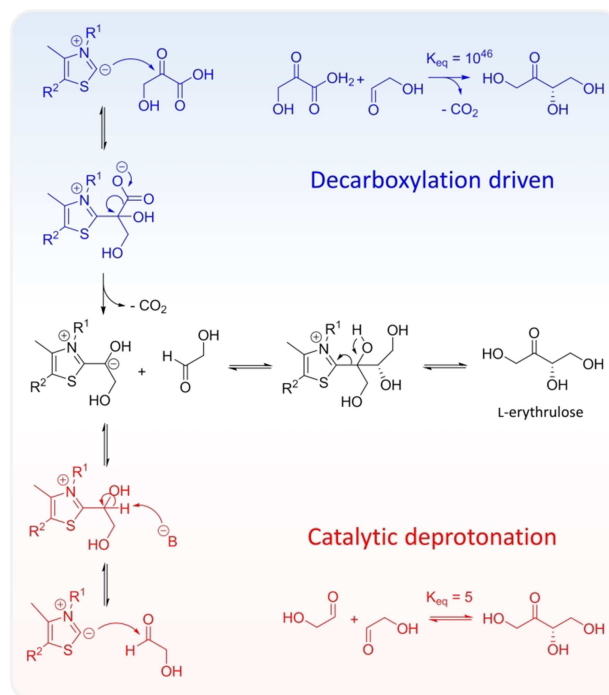
outside of the scope of this review and more extensive discussions on this topic can be found elsewhere.^[21,23]

Thiamine Diphosphate Dependent Enzymes

Thiamine diphosphate (ThDP) dependent enzymes catalyse the synthesis of chiral α -hydroxyketones (acyloins), which is an important structural motif in many high-value carbohydrates and pharmaceuticals. The reaction comprises of an asymmetric carbon-carbon bond forming step between two aldehyde substrates, of which the donor substrate is rendered a nucleophile following an 'Umpolung' mechanism with the ThDP cofactor. To afford the catalytically active ylide state, the enzyme must stabilise the cofactor in an energetically disfavoured V-conformation that is evolutionarily conserved within this enzyme class.^[25] While ThDP dependent enzymes typically convert a large variety of aliphatic-,^[26] aromatic,^[27] and hydroxyaldehydes^[28] as acceptor substrates, they generally have a more strict specificity towards relatively few donor substrates.

The ThDP dependent enzyme transketolase (TK) is a key enzyme of the pentose phosphate pathway, where it catalyses the reversible transfer of a C2-ketol group from a ketose to an aldose sugar. For synthetic applications, hydroxypyruvate (HPA) is used as donor substrate, since irreversible decarboxylation directly affords the carbanion in the α,β -dihydroxyethylthiamine diphosphate (DHETHDP) intermediate (Scheme 4, top). In contrast, catalytic deprotonation is required for the formation of the nucleophilic carbanion when glycolaldehyde is being used as the analogous ketol donor without decarboxylation (Scheme 4, bottom). At this stage, both the kinetically and the thermodynamically controlled synthesis of L-erythrulose share the same covalent intermediate DHETHDP, the same mode of C–C bond formation and mechanism of product release.

Notably, the reverse splitting of L-erythrulose into two molecules of glycolaldehyde occurs with 100% atom economy. ThDP catalysed reactions therefore constitute a particularly interesting case of kinetic control, where the reverse reaction is



Scheme 4. Decarboxylation driven synthesis of L-erythrulose from hydroxypyruvate and glycolaldehyde under kinetically controlled conditions (top) and the thermodynamically controlled one-substrate synthesis from two molecules of glycolaldehyde (bottom). The two reaction pathways share a common intermediate and mechanism of product release (middle), which mechanistically interconnects both types of reaction. Therefore, the product concentration ultimately converges towards the thermodynamic equilibrium of the one-substrate reaction upon dissipation of the initial driving force. For simplicity reasons, the pyrimidine and pyrophosphate groups of the ThDP cofactor are abbreviated as R¹ and R².

an intrinsic property of the product (e.g. the reverse hydrolysis of esters can be circumvented in the absence of water).

The kinetically controlled synthesis of L-erythrulose displays a high (k_1/k_2) ratio and full thermodynamic equilibration was only observed over extended time periods of several weeks.^[26a] The high ratio of (k_1/k_2) previously resulted in inaccurate reports, where product formation was falsely being described as irreversible.^[26b,28b, 9] Based on the reversible splitting of L-erythrulose, an amperometric biosensor has since been developed for the quantitation of ThDP in clinical samples. While ThDP catalysed reactions can be essentially irreversible from a practical point of view, this application illustrates the importance of accurately describing similar reactions as being kinetically controlled.^[30]

Glycosyl Transferases

Glycosyl transferases catalyse the stereoselective synthesis of glycosidic bonds in (poly-) saccharides. In the case of LeLoir glycosyl transferases, activated nucleotide sugars serve as glycosyl donors. The high kinetic stability of glycosidic bonds with respect to non-catalytic hydrolysis makes them a particularly interesting example for the study of thermodynamically

and kinetically controlled reactions. The hydrolysis of trehalose into two molecules of glucose is catalysed by the enzyme trehalase (Scheme 5). Conversely, trehalose transferase (TreT) does not exhibit any significant hydrolysis activity and therefore displays a near ideal synthesis to hydrolysis ratio with $k_1 \gg k_2$.

As described above for acyl transferases, the choice of activated donor substrate plays an important role for the overall performance. For glycosyl transferases, the specific choice of activated nucleotide sugar used for the coupling, such as uridine diphosphate (UDP) or adenosine diphosphate (ADP) strongly influences the extent of conversion. In contrast to UDP-glucose, the use of ADP-glucose as glycosyl donor releases insufficient free energy during the elimination of ADP to facilitate a shift of the equilibrium constant towards full conversion.^[12] Albeit kinetically controlled, the reaction therefore also remains limited by thermodynamics when ADP-glucose is used as glycosyl donor.

Kinases

Thermodynamically and kinetically controlled (de-) phosphorylation reactions play a central role in biological systems, where they are used for both the activation of biomolecules and in signalling cascades. For example, glycolysis is initiated by the hexokinase catalysed phosphorylation of glucose using ATP. The requirement for phosphorylated substrates during several steps in central metabolism constitutes an elegant regulatory control mechanism.^[28b] The kinetically controlled phosphorylation of molecules by kinases functions as an activator, while the thermodynamically preferred hydrolysis of phosphate esters results in the termination of activity. In the context of biocatalytic conversions, kinases have been extensively used to produce phosphorylated reactants *in situ*.^[31] While most kinases use ATP as the preferred phosphate donor, the relatively high price of ATP renders its regeneration from cheaper phosphate sources essential for the economic feasibility of large scale applications. Enzymatic systems for the regeneration of ADP

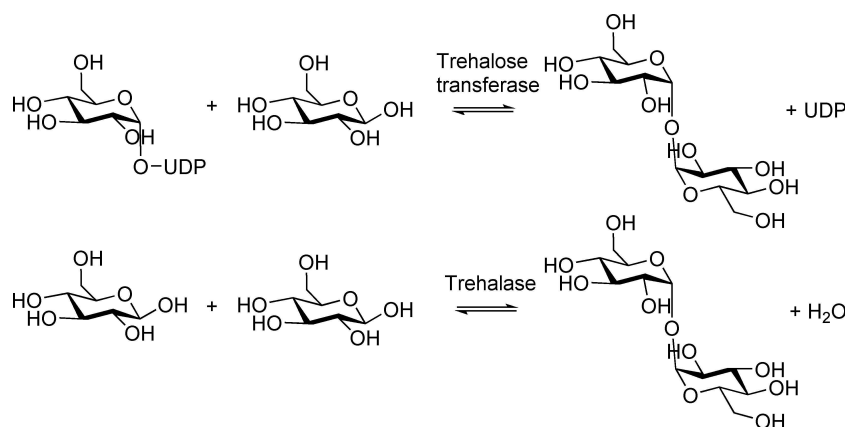
and ATP have therefore extensively been studied.^[31,32] An overview of these systems using various phosphate donors is summarised in Scheme 6.

Quantitative ³¹P-NMR has been demonstrated to be a particularly useful method for the detailed study of the kinetically controlled phosphorylation of D-glyceraldehyde by dihydroxyacetone kinase (DHAK). This phosphorylation reaction was followed over time, allowing for the simultaneous monitoring of substrate conversion, product formation (D-glyceraldehyde phosphate) and its rate of hydrolysis *via* the formation of inorganic phosphate.^[33] This reaction is an elegant example of kinetic vs thermodynamic control in that both the regeneration of ATP and its subsequent use in phosphorylation reactions compete with reverse hydrolysis. This infers that kinetic control is a necessity for both steps due to the unfavourable thermodynamic equilibrium of these reactions *in aqua* (Schemes 6 and 7). Given this is one example, the field of kinases clearly warrants further examination in the context of reaction control and limitations.

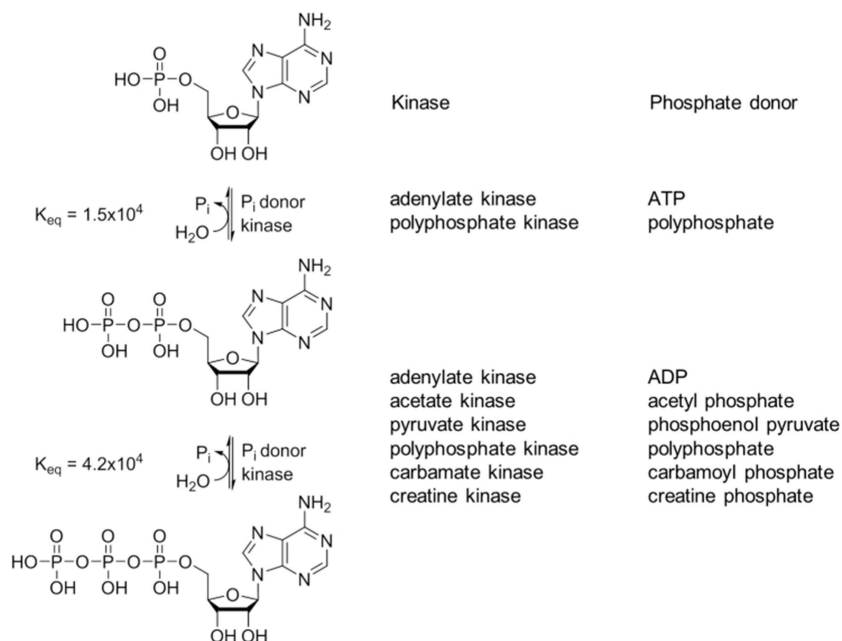
Thermodynamically and Kinetically controlled Enzymatic Synthesis of Diastereomers

Enzymes are excellent catalysts that naturally evolved to catalyse chemical conversions with great selectivity and high enantiomeric excess. This is achieved by means of ground state destabilisation, conformational substrate stabilisation, enzyme preorganisation and the stabilisation of transition states through the precise control of spatial arrangements within the active sites.^[35] For reactions which start from a prochiral molecule, the two product enantiomers possess the same free energy of formation and the enantiomeric excess remains constant throughout the course of reaction as an intrinsic property of the active site geometry.

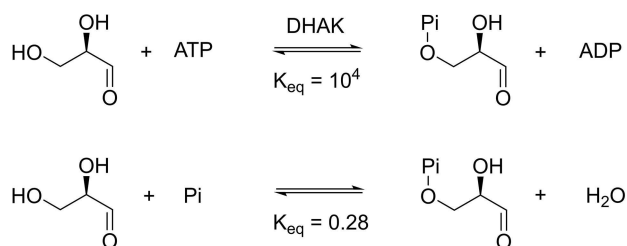
In contrast, diastereomers do not possess the same free energy of formation, which amongst many other properties,



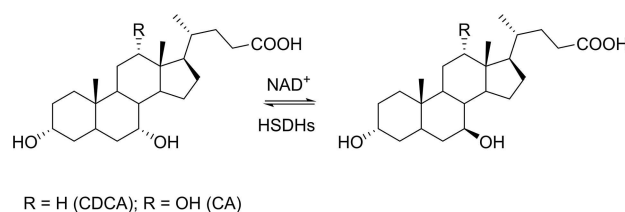
Scheme 5. Trehalose transferase catalysed synthesis of trehalose from a nucleotide sugar (e.g. UDP-glucose) and glucose as acceptor (top) and reverse hydrolysis of trehalose back into the corresponding monomers in aqueous solution (bottom). In nature, the reverse reaction is catalysed by the enzyme trehalase.



Scheme 6. Overview of selected kinases and phosphate donors for the successive phosphorylation of AMP *via* ADP to ATP.^[31,32] Equilibrium constants are given for hydrolysis in the reverse direction, which is thermodynamically favoured.



Scheme 7. Dihydroxyacetone kinase (DHAK) catalysed phosphorylation of D-glyceraldehyde with ATP to D-glyceraldehyde-3-phosphate (G3P) and ADP. Spontaneous hydrolysis of G3P to D-glyceraldehyde and phosphate, with an approximate half-life of 24 hours at pH 6.7 and 298 K.^[33]



Scheme 8. Thermodynamic epimerisation of the 7-hydroxyl group in cholic acid (CA) or chenodeoxycholic acid (CDCA) by application of two stereocomplementary hydroxysteroid dehydrogenases (7 α - and 7 β HSDHs) in a redox neutral fashion. An energy difference of -1.23 kJ/mol under standard conditions was calculated for the two diastereomers ($K_{eq} = 1.64$), which acts as the sole driving force for epimerisation.^[38]

allows for their discrimination (e.g. by chromatography or NMR). Most notably, the existence of an energy difference $\Delta\Delta G$ between two diastereomers determines their corresponding equilibrium distribution (i.e. diastereomeric excess) according to Equation (1), provided that the reaction conditions allow for their mutual interconversion.

This concept has been elegantly applied in the thermodynamic epimerisation of hydroxysteroids for the production of ursodeoxycholic acid, an active pharmaceutical ingredient that is being used for the treatment of cholestatic diseases.^[36] A combination of two enantiocomplementary hydroxysteroid dehydrogenases (7 α - and 7 β HSDHs, Scheme 8) was used to chemically interconnect both diastereomers *via* the corresponding ketone in a redox neutral fashion, and conversions in excess of 90% towards the target diastereomer were achieved with their energy difference acting as the sole driving force.^[37]

However, in cases where the target diastereomer does not constitute the thermodynamic product, its synthesis then

effectively needs to be kinetically controlled to obtain a high diastereomeric excess. The thermodynamically and kinetically controlled synthesis of diastereomers has extensively been studied for Diels-Alder reactions since their discovery in 1929,^[7] and has remained the topic of extensive studies until today.^[8] In this type of reaction, the conversion selectively proceeds through the lowest energy transition state at low temperatures, whereas higher temperatures allow for it to proceed through both transition states and therefore to equilibrate towards the formation of the thermodynamic product (Figure 2).

In the following, parallels are drawn between the energy diagram of Diels-Alder reactions (Figure 2) and that for the enzymatic synthesis of diastereomers (Figure 3). For simplicity reasons, the introduction of one new chiral centre to afford two diastereomers will be discussed at the example of a hypothetical reaction, that is catalysed by a single enzyme and affords the (*S*) configuration at the newly formed stereocentre as the thermodynamic product (Figure 3).

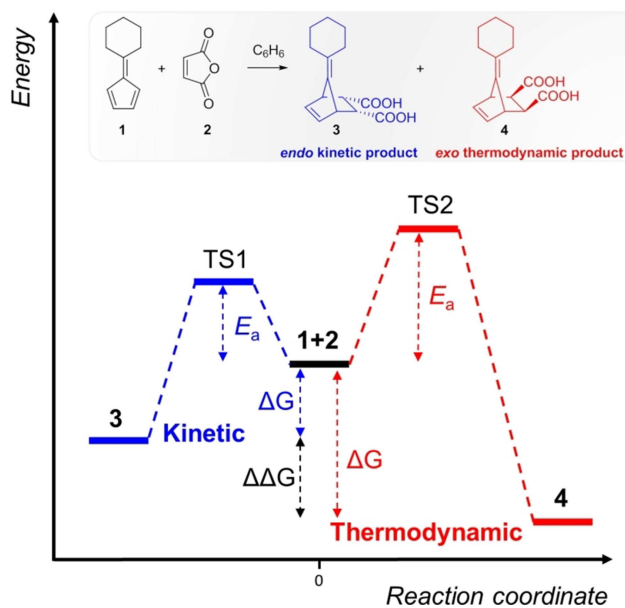


Figure 2. Qualitative energy diagram for the conversion of fulvene **1** and maleic anhydride **2** and in a Diels-Alder reaction to selectively afford the *endo* **3** and *exo* **4** configured products under either thermodynamically or kinetically controlled conditions. Low temperatures favour the reaction pathway which proceeds through the lower energy transition state, while high temperatures allow the reaction to proceed through the higher energy transition state to afford the thermodynamic product.^[7a] The equilibrium distribution of diastereomers **3** and **4** under thermodynamic conditions is determined by the corresponding energy difference according to Equation (1).

In spite of their generally high stereoselectivity, enzymes are not perfect catalysts and therefore, to some extent, catalyse the formation of both stereoisomers. Since most enzymatic reactions are mechanistically reversible, the product diastereomers are rendered dynamically interconnected, which is illustrated in Figure 3. The equilibration towards the thermodynamic product was previously already demonstrated for the mechanistically interconnected, kinetically controlled synthesis of L-erythrulose using the enzyme transketolase.^[26a]

Due to the asymmetry of active sites, the *pro-R* and *pro-S* transition states are stabilised by different chemical environments and thereby also display different activation energies (E_a).^[35] By comparison, the enzymatic synthesis of diastereomers shows the same characteristic energy diagram as Diels-Alder reactions (Figure 2, Figure 3). In cases where the (*S*) configured diastereomer constitutes the thermodynamic product, an (*R*)-selective enzyme will initially afford the (*R*) configured product under kinetically controlled conditions. If the reaction is allowed to proceed, subsequent epimerisation into the (*S*) diastereomer will occur and establish the corresponding thermodynamic equilibrium distribution of diastereomers based their difference in free energy. Similarly, an (*S*) selective enzyme will initially afford the (*S*) configured product under kinetically controlled conditions with high stereoselectivity as a reflection of the enzyme's active site geometry. Nonetheless, prolonged reaction times will establish the same distribution of diastereomers for both an (*R*) or (*S*) selective enzyme under thermodynamic

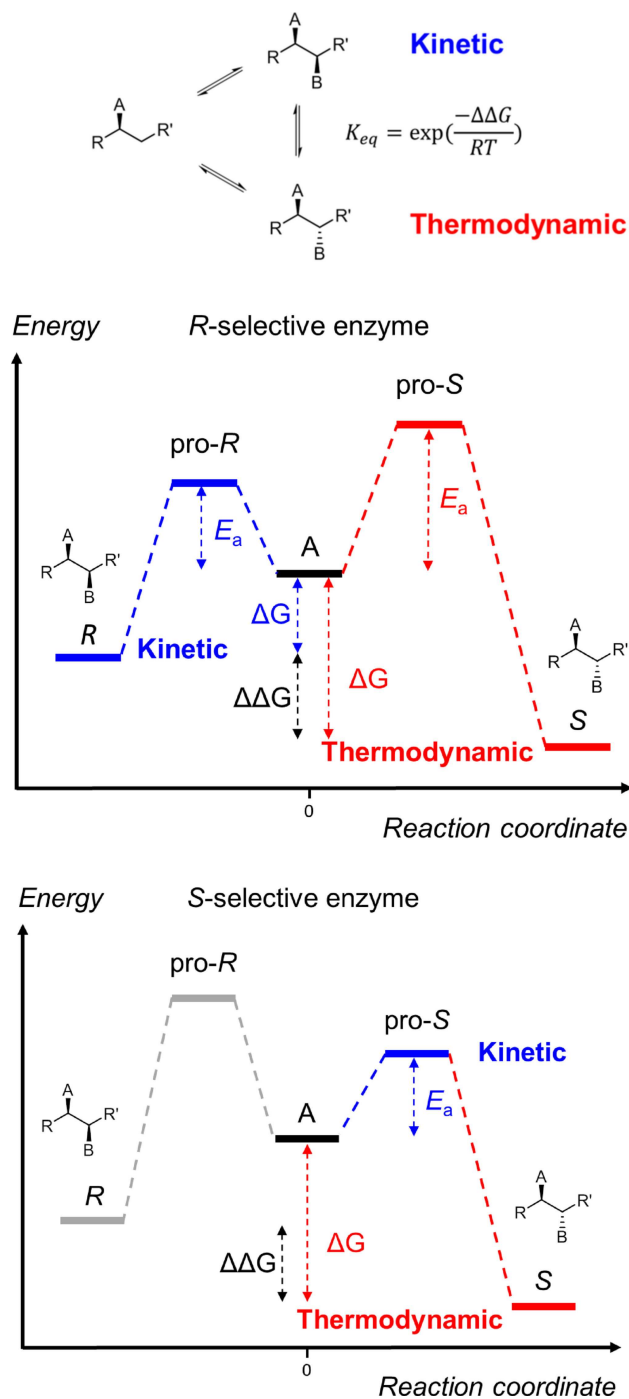


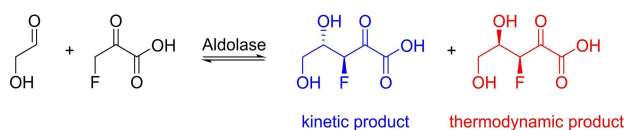
Figure 3. Qualitative energy diagrams for a hypothetical reaction, in which the diastereomers with the newly formed (*S*)-stereocentre is lower in energy than the (*R*) diastereomer. A dynamic equilibrium is established between both diastereomers by the mechanistically reversible nature of catalysis (top). Using an (*R*)-selective enzyme (middle) affords the *R*-configuration as the kinetic product, which subsequently equilibrates towards the (*S*) configured product under thermodynamically controlled conditions. With an (*S*)-selective enzyme (bottom), the (*S*) diastereomer is directly formed as the kinetic product. Eventually, the diastereomeric excess will be identical for both enzymes according to $\Delta\Delta G$ when thermodynamically controlled conditions are established.

conditions. Thus, the diastereomeric excess will also decrease over time for an (*S*) selective enzyme.

For example, the conversion of fluoropyruvate by a variety of type I and type II aldolases initially affords a single product with high diastereomeric excess, but this value was observed to decrease over time and the reactions needed to be kinetically controlled (Scheme 9).^[38] In another study, lower enzyme loadings increased the diastereomeric excess under otherwise identical conditions at the expense of conversion.^[39]

Thus, the absolute configuration at the newly formed chiral centre(s) does not remain constant and changes over time towards that of the thermodynamic product. A qualitative progression curve of the absolute configuration for the previously discussed hypothetical reaction is shown in Figure 4. Both the position of the final equilibrium and the rate of progression towards it strongly depend on the specific reaction parameters (temperature, pH, enzyme loading). Single measurements of the diastereomeric excess therefore only allow to a limited extent for conclusions regarding the enzyme's stereoselectivity. This could even lead to a falsely assigned stereoselectivity for the enzyme, as the actual position within this progress curve remains unknown.

Formerly, biocatalytic conversions were typically carried out within a narrow temperature range close to physiological conditions, due to the frequent temperature sensitivity of microorganisms and wild-type enzymes. These conditions generally favour the synthesis of kinetic products. With the



Scheme 9. Aldol coupling of fluoropyruvate and glycolaldehyde, catalysed by three different promiscuous class II pyruvate aldolases (*EcGarL*, *EcRhmA*, *EcHpCh*). The *anti*-configured product is obtained with high diastereomeric excess (71–93 %) at low catalyst loadings (0.01 mol%), while only a low to moderate diastereomeric excess (39–59 %) is obtained with a higher catalyst loading (0.05 mol%) under otherwise identical conditions.^[39]

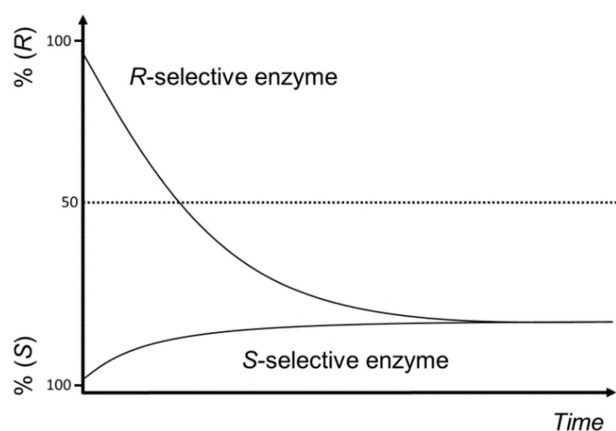


Figure 4. Progression of the absolute configuration at the newly formed chiral centre over time for the hypothetical reaction discussed in Figure 3.

emergence of computational algorithms to enhance the thermostability of enzymes,^[40] and the plethora of genomes that are currently available from thermophilic organisms, biocatalytic conversions are becoming increasingly compatible with elevated reaction temperatures.^[41] As a consequence, reactions are increasingly at risk to also proceed through higher energy transition states, which circumvents kinetic control and more rapidly establishes the thermodynamic equilibrium distribution of products.

While it is easy to conceptualize transition states as well-defined structures, *transition state landscapes* better capture their dynamic and structurally diverse nature.^[42] A detailed QM/MM analysis of enzymes belonging to the alkaline phosphatase superfamily described the stabilisation of multiple loose transition states within single active sites.^[43] This study found that alkaline phosphatases are able to recognise and stabilise multiple transition states without undergoing larger structural rearrangements. This suggests that elevated reaction temperatures could similarly also impact an enzyme's regioselectivity by allowing conversions to proceed through more distant transition states that are higher in energy. Elevated reaction temperatures could therefore potentially impact the outcome of many reactions, ranging from a switch in absolute configuration, to the formation of entirely new products as a result of a changed regioselectivity.

Conclusions

Using the elimination of a good leaving group in substrate analogues as driving force, kinetically controlled conditions enable enzyme catalysed conversions which otherwise would not be economically viable under thermodynamically controlled conditions. While the use of substrate analogues comes at the cost of a lower atom economy, this is generally outweighed by the benefits that come with increased levels of conversion and improved chemo-, regio- and enantioselectivity. In this way, kinetically controlled reactions have found widespread applications for the synthesis of chiral building blocks and high value chemicals on large scale.

The enzyme catalysed synthesis of diastereomers shows the same characteristic energy diagram as that for Diels-Alder reactions and enzymatic conversions similarly proceed initially through the lowest energy transition state at low temperatures.

However, enzymes are not perfect catalysts and therefore mechanistically interconnect the synthesis of both diastereomers. Ultimately, enzymes establish the equilibrium distribution of diastereomers that is intrinsically determined by their difference in free energy. While this may be desirable in cases like the thermodynamic epimerisation of hydroxysteroids, the target molecule does not necessarily always constitute the thermodynamic product, and its synthesis then needs to be kinetically controlled. While the absolute configuration of the newly formed chiral centre(s) initially is determined by the enzymes stereoselectivity under kinetically controlled conditions, thermodynamic equilibration can lead to the inversion of the

stereocentre. Single measurements of the diastereomeric excess therefore only provide limited information and could even lead to an enzyme's stereoselectivity being falsely assigned. This notion is of particular relevance, as biocatalytic conversions are increasingly conducted at elevated temperatures; conditions which are known to favour the formation of thermodynamic products.

Acknowledgement

The authors would like to thank all group members, past and present that have participated in and contributed to our research effort. L. M. is grateful for the generous sponsoring via the ERA-IB scheme, grant ERA-IB-15-110.

Conflict of Interest

The authors declare no conflict of interest.

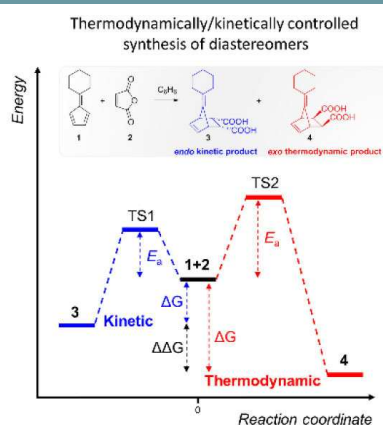
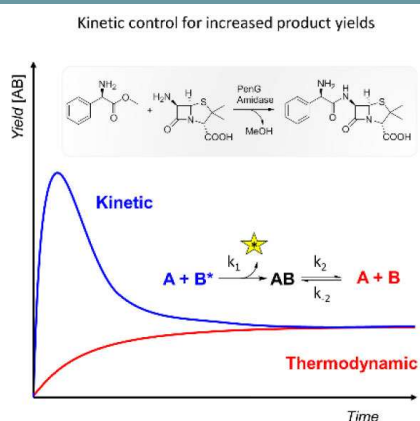
Keywords: Thermodynamic control · Kinetic control · Diastereomers · Asymmetric synthesis · Biocatalysis

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Manuscript received: August 26, 2019
 Revised manuscript received: November 1, 2019
 Accepted manuscript online: November 4, 2019
 Version of record online: ■■■, ■■■■

MINIREVIEWS



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Hanefeld*

1 – 13

Thermodynamically and Kineti-
cally Controlled Reactions in Bio-
catalysis – from Concepts to Per-
spectives

Biocatalysis: Kinetic control usually is applied in biocatalysis to improve product yields under thermodynamically challenging conditions, and the use of substrate analogues then allows for (near) complete conversion.

The enzymatic synthesis of diastereomers shows an energy diagram similar to that of Diels-Alder reactions and the same concepts of thermodynamic and kinetic control also apply to enzyme catalysis.

