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Thermodynamics Determine the Diastereochemical Outcome of Catalytic Reactions

Stefan R. Marsden,[a] Luuk Mestrom,[a] Hein J. Wijma,[b] Sander J. Noordam,[a]
Duncan G. G. McMillan,[a] and Ulf Hanefeld*[a]

Diastereomers are characterised by an intrinsic energy difference, and thermodynamics dictate their distribution within a dynamic equilibrium. The characteristic mechanistic reversibility and non-ideal stereoselectivity of catalysts therefore simultaneously promote both synthesis and epimerization of products during the formation of diastereomers. This feature can even result in the thermodynamic inversion of a chiral centre against the catalyst's stereoselectivity. Here, we provide a comprehensive experimental and theoretical study of factors that govern thermodynamic epimerization in catalysis, using enzymes as example. Our analysis highlights, that the deduction of a catalyst's stereoselectivity based on the absolute configuration of the isolated product constitutes a potential pitfall. The selective formation of either the thermodynamic-, or the kinetic product is less determined by the catalyst, but rather by the reaction conditions. Next to low temperatures, a high maximal extent of conversion was identified to promote kinetically controlled conditions. For bimolecular reactions, conversions can be conveniently modulated via the use of one substrate in excess. Quantum mechanical calculations accurately predicted the diastereomeric excess under equilibrium conditions, which opens the prospect of a rational choice between thermodynamic and kinetic reaction control at an early stage of process design. Our findings are of critical importance for multi-step syntheses of stereocomplex molecules via catalytic cascade reactions or artificial metabolic pathways, as the final stereochemistry may be determined by the absolute configuration of the product that is overall lowest in energy.

Introduction

Biological systems are inherently chiral due to the unique structures of DNA, proteins and metabolites, and two enantiomers of the same compound often display different biological properties.[1] This notion renders the development of catalysts that display a high chemo-, regio- and enantioselectivity fundamentally important for applications within life sciences. Kinetic reaction control is a widely prevalent approach in organic synthesis to modulate the product selectivity of a reaction. It is conceptually based on the faster formation of a reaction intermediate (i.e. the kinetic product), over formation of the lowest energy (i.e. thermodynamic) product.[2] Continuous monitoring of the reaction's progress is essential to

circumvent equilibration toward the most stable product under thermodynamically controlled conditions.[3] For instance, the stereoselective synthesis of enantiomers is kinetically controlled, while the racemic mixture constitutes the thermodynamic product.[4] Enantiomers possess the same free energy of formation ($\Delta G = 0$), and an increase in entropy promotes their racemization ($K_{eq} = 1$, $ee\% = 0$) according to equation (1).[5] Conversely, diastereomers are characterised by an intrinsic energy difference; this feature determines their distribution within a dynamic equilibrium. This is for instance the case with readily interconverting conformational isomers, and their irreversible conversion following Curtin-Hammet/Winstein-Holness (CH/WH) type kinetics has been extensively investigated since the 1950s.[6] The CH/WH principle states, that for $k_{AC}, k_{BD} \gg k_{AB}, k_{BA}$, the product distribution of C,D reflects the initial distribution of diastereomeric conformers A,B (Figure 1a). The irreversible nature of reactions that are described by CH/WH type kinetics renders these reactions kinetically controlled and prevents their subsequent thermodynamic equilibration. For the reverse case $k_{AB}, k_{BA} \gg k_{BD}, k_{AC}$, the distribution of CD is also constant and determined by k_{BD}, k_{AC}.

$$\Delta G = -RT\ln K_{eq} \qquad (1)$$

In contrast, mechanistically reversible, non-catalytic Diels-Alder reactions afford the product diastereomers within a dynamic equilibrium.[7] This allows for the selective isolation of either the thermodynamic or the kinetic reaction product via modulation of the process conditions.[8] At low temperatures, Diels-Alder reactions selectively proceed through the lowest energy transition state to afford the kinetic product, while

[a] S. R. Marsden, Dr. L. Mestrom, S. J. Noordam, Dr. D. G. G. McMillan, Prof. U. Hanefeld
Biokatalyse, Afdeling Biotechnologie
Technische Universiteit Delft
van der Maasweg 9
2629HZ Delft (The Netherlands)
E-mail: u.hanefeld@tudelft.nl
[b] Dr. H. J. Wijma
Groningen Biomolecular Sciences and Biotechnology Institute
Faculty of Science and Engineering
University of Groningen
Nijenborgh 4
9747AG Groningen (The Netherlands)

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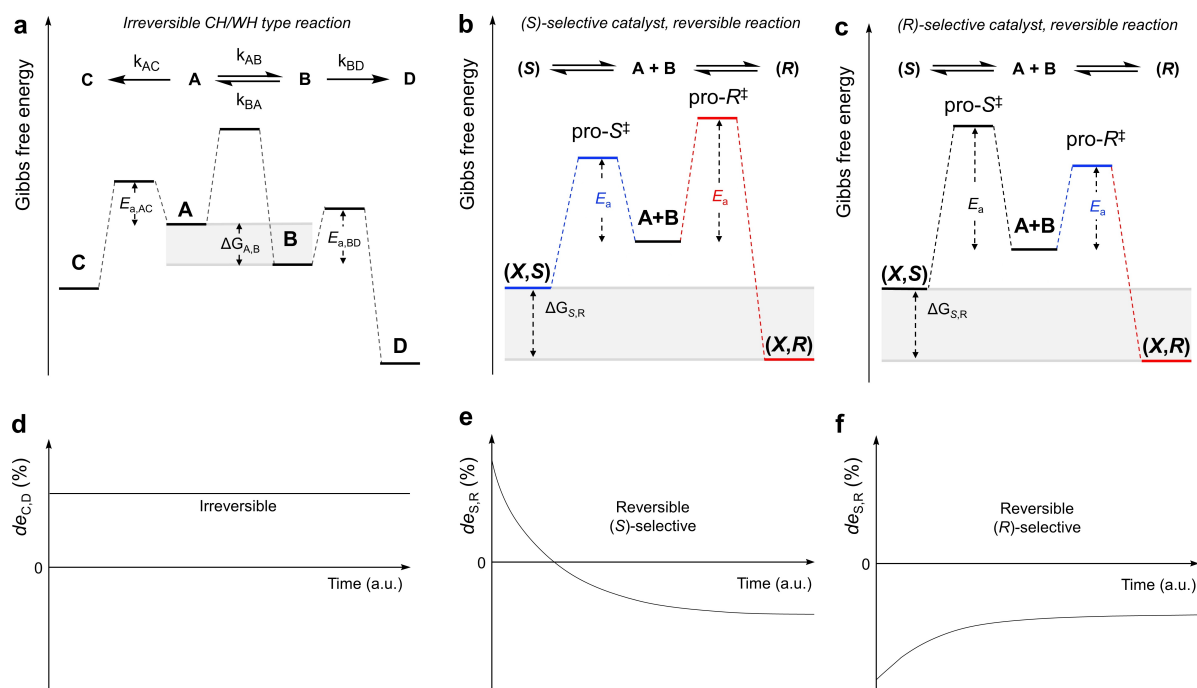European Chemical
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Figure 1. Qualitative energy diagrams and related diastereomeric excess time courses. a,d) Curtin-Hammett type irreversible conversion of interconverting conformers AB. For $k_{AC}, k_{BD} \gg k_{AB}, k_{BA}$, the distribution of CD is constant and reflects the initial distribution of A and B. b,c) Hypothetical reaction, in which the (X,R)-configured diastereomer constitutes the thermodynamic product, catalysed by either (X,R)- or (X,S)-selective catalysts. The second stereocentre (X, for diastereomers required) is fixed and can be either (R)- or (S)-configured. The catalyst's stereoselectivity is reflected by a lower activation energy E_a. e,f) The kinetic product is initially afforded according to the catalyst's diastereoselectivity. Simultaneous epimerization ultimately establishes the same thermodynamic distribution of diastereomers according to $\Delta G_{R,S}$. For (S)-selective catalysts, this inverts the stereochemical outcome (e). For (R)-selective catalysts, it reduces the diastereomeric excess (f).

prolonged reaction times and elevated temperatures result in the formation of the thermodynamic product.[7–8] The diastereomeric excess (de) is then based on the corresponding energy difference between the diastereomers. Analogous to non-catalytic Diels-Alder reactions, the diastereoselectivity of a catalyst can be translated into a similar energy diagram (Figure 1b,c).[3] For simplicity reasons, the second stereocentre (X), that is required for a molecule to be a diastereomer, is considered to be fixed as either (R)- or (S)-configured. However, the discussed concepts similarly apply to catalysts that display no enantioselectivity, and therefore afford (X) in both configurations. In this interpretation, the activation energies E_a of the corresponding pro-(R) and pro-(S) transition states reflect the catalyst's diastereoselectivity.[9]

The characteristic mechanistic reversibility and non-ideal stereoselectivity of catalysts then establish a dynamic equilibrium between the different product diastereomers. Catalysts therefore simultaneously promote both the synthesis and epimerization of products during the formation of diastereomers. This is conceptually similar to Diels-Alder reactions, and opposite to irreversible, CH/WH type systems. Under thermodynamic conditions, the stereochemical outcome for the catalytic synthesis of diastereomers is therefore independent of the catalyst, and may be opposite to its stereoselectivity.

Declaredly, epimerization effects were previously reported at the specific example of threonine aldolases.[10] Temperature and ratio of substrates were described to have a strong

influence on the outcome of the reaction, indicating the influence of thermodynamics on the diastereoselectivity. Yet, surprisingly, similar studies with other enzymes and a broader discussion of thermodynamic epimerization effects in catalysis, such as their implications for multi-step cascade reactions, remain largely absent in literature.

Here, we present a theoretical and experimental analysis of general factors that influence the extent of thermodynamic epimerization, such as temperature, catalyst stereoselectivity, free energy difference between product diastereomers and the particular stoichiometry of substrates. Quantum mechanical methods were applied to predict the identity and associated diastereomeric excess of the thermodynamic product under equilibrium conditions. Finally, catalytic single and two-step conversions were used as model systems to study the effect of thermodynamic epimerization for multi-step cascade reactions. In this manner, we aim to provide a comprehensive overview of thermodynamic epimerization effects for catalysis.

Results and Discussion

Time course studies

The stereocomplementary hydroxyketoacid aldolases from *Sphingomonas wittichii* (*Sw*HKA) and *Burkholderia phytofirmans* (*Bp*HKA) selectively catalyse an aldol reaction between fluoro-

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pyruvate as the donor substrate, and a wide variety of aldehydes as acceptor substrates.[11] This conversion creates two chiral centres in one step from achiral substrates, circumventing possible interferences from a (dynamic) kinetic resolution of the substrates. This makes it an ideal system for monitoring thermodynamic epimerization in a time-related process (Figure 2). The extent of conversion and absolute configurations were both quantitatively monitored over time by ^{19}F-NMR. At low levels of conversion, spectra in which the signal-to-noise ratio of the minor product peak was below 2:1 were discarded, in order to reliably determine the diastereomeric excess in all cases.

Stereoselective reduction of the ketoacid aldol products with L-lactic dehydrogenase was applied to differentiate the enantiomers by their conversion into unique diastereomers. This allowed to determine both the enantiomeric excess, and to assign the absolute configuration for the fluorine moiety via its $^{3}J_{HF}$ coupling constant to the newly formed stereocentre (Figure S7). In agreement with previous reports,[11–12] both SwHKA and BpHKA were found to be highly (3S)-selective, and the minor (3R)-configured enantiomers were below the limits of reliable detection under the conditions examined. However, both enzymes displayed a moderate diastereoselectivity with respect to the configuration of the C-4 hydroxyl group. Anti selective SwHKA (3S,4S) and syn selective BpHKA (3S,4R) initially yielded aldol products according to their stereoselectivity at low levels of conversion. Strikingly, both enzymes simultaneously also catalysed the thermodynamic epimerization of the aldol products toward the corresponding equilibrium distribution of syn and anti configured diastereomers (Figure 2). The reaction profile of SwHKA therefore corresponds to the qualitative energy diagram shown in Figure 1b, with the corresponding diastereomeric time course shown in Figure 1e. Similarly, reactions with BpHKA correspond to the cases shown

in Figures 1c,f. Since both SwHKA and BpHKA are highly (3S)-selective, syn/anti epimerization was exclusively observed at the position of the C-4 hydroxyl group. Having made that observation, it is worthy to note that the diastereomeric excess also decreased over time in the case of BpHKA, which is selective for the formation of the thermodynamic product.

In silico modelling

With a straightforward protocol for the study of thermodynamic epimerization effects in hand, we set out to model the impact of different parameters on the catalytic system. Under thermodynamic conditions, the diastereomeric excess is determined by the energy difference between product diastereomers according to equation (1). Strikingly, a $\Delta G \geq 13.2$ kJ/mol is required to achieve a diastereomeric excess of $>99\%$ at 298 K (Figure S10). Adequate optical purities may therefore not be achievable under thermodynamic conditions for a multitude of molecules. The stereoselectivity **s** of a catalyst is one of the most important catalytic descriptors, and is derived from the ratio of forward rate constants k_R and k_S, that lead to the corresponding (R)- and (S)-configured products.[13] For enzymes, the enantioselectivity has been described as E,[14] while the diastereoselectivity has not yet been discussed as a separate parameter. In this manner, **s** correlates with the maximal optical purity of the kinetic product. Yet, even highly selective catalysts simultaneously promote product epimerization, and ultimately afford the same diastereomeric excess under thermodynamic conditions (Figure 3a).

The equilibrium constant (K_{eq}) for any type of reaction is set by the temperature and the free energy difference between substrates and products according to equation (1). K_{eq} further correlates with the ratio of apparent rate constants for the
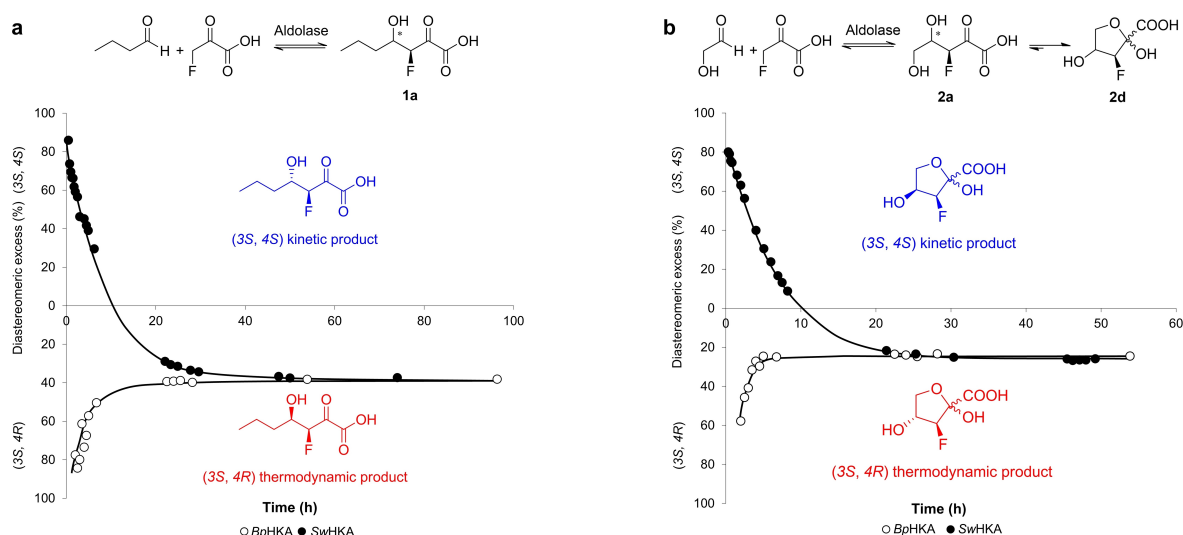

Figure 2. Enzyme catalysed aldol reaction between fluoropyruvate and butyraldehyde (a) or glycolaldehyde (b). SwHKA is anti selective (3S,4S), but simultaneously catalyses the epimerization into the syn (3S,4R) configured thermodynamic product. BpHKA is selective for the thermodynamic product (syn selective, 3S,4R), but similarly catalyses the epimerization toward the thermodynamic equilibrium distribution of both diastereomers, reducing the diastereomeric excess over time.
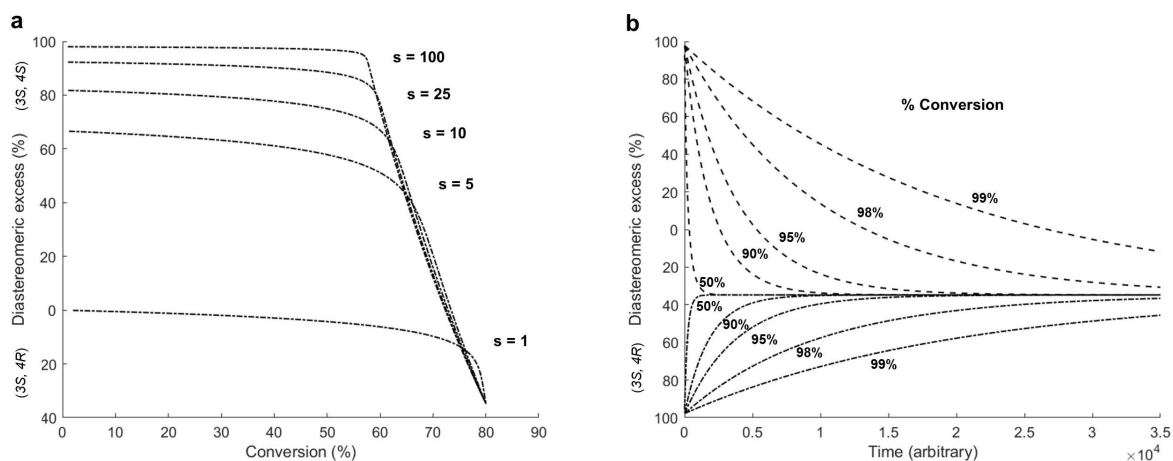
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Figure 3. a) Model for the aldol reaction between fluoropyruvate and butyraldehyde with hypothetical catalysts of different diastereoselectivity **s**, which determines the maximal optical purity of the kinetic product, but even high **s** values do not suppress epimerization. b) Higher levels of conversion (equivalent to a high K_{eq}) increase the ratio of rate constants for the forward and reverse reaction (k_f/k_r), which kinetically disfavours epimerization. The system therefore converges toward CH/WH type features with increasing maximal extent of conversions. Model parameters: $\Delta G_{RS} = 1.8$ kJ/mol, **s** = 100, 298 K. For more details, see pages S21–S23 in the supporting information.

forward and reverse reaction (k_f/k_r). The reverse reaction therefore becomes kinetically less significant with an increasing maximal extent of conversion; ultimately converging toward the properties of an irreversible CH/WH type system (Figure 1d, 3b, Figure S12). For bimolecular reactions, the use of one substrate in excess can additionally be applied to drive conversions toward the product side. Based on our model, the ratio of forward to reverse rate constants appears to be the most important parameter for simultaneous product epimerization during the synthesis of diastereomers.

Experimental validation

In order to validate the results from our model, the catalyst loading was initially varied. This demonstrated that the observed epimerization of diastereomers was indeed enzyme catalysed and proceeded against the catalyst's stereoselectivity. Notably, the diastereomeric excess of the product mixture was found to be independent of the reaction time and catalyst loading, correlating only with the extent of conversion (Figure 4a). This observation has significant implications from both an economic and an environmental perspective, where high conversions are desirable.[15] Maximal conversions involve a concomitant loss of stereocontrol by the catalyst, as the reaction converges toward the thermodynamic equilibrium (Figure 4a). Here, the use of one substrate in excess may remedy this drawback. In agreement with our model, the addition of up to four equivalents of butyraldehyde did not only increase the overall conversion, but also reduced epimerization of the kinetic product (Figure 4b). While solubility limitations prevented us from further increasing the substrate loading, enzymes from other classes, such as transaminases, are commonly applied with one substrate in excess,[16] which can be expected to increase the duration of kinetically controlled

conditions accordingly. This was also observed in the aforementioned study of threonine aldolases.[10g] Similar to non-catalytic Diels-Alder reactions, variation of the reaction temperature should allow for a switch from thermodynamically to kinetically controlled conditions and *vice versa*. Indeed, low temperatures were found to increase the selectivity toward formation of the kinetic product, whereas elevated temperatures generally promoted thermodynamic epimerization (Figure 4c). Notably, the C-4 stereoselectivity of *Sw*HKA remained similar over the tested temperature range (0–40 °C, **s** ≈ 9 corresponding to a *de* ≈ 80%). The increase in selectivity toward formation of the kinetic product (Figure 4c) therefore seems to arise from a significantly lower rate for the reverse reaction in the retro-aldol direction, which is essential for product epimerization. Aldol reactions with glycolaldehyde generally displayed a higher selectivity toward formation of the kinetic product, and the effect of temperature on the extent of epimerization was notable, but less pronounced (Figure 4d). Different temperatures therefore impact the position of the thermodynamic equilibrium, but do not circumvent epimerization. While reactions with aliphatic aldehydes comprise of only one step (namely the aldol reaction, Figure 4c), reactions with hydroxyaldehydes comprise of two consecutive equilibrium reactions (the aldol reaction, followed by a spontaneous intramolecular cyclisation, Figure 4d). This increases the total free energy change of reaction and thereby also the overall extent of conversion. Reaction systems which allow for a high level of conversion by means of follow-up reactions are therefore similarly less prone to thermodynamic epimerization. However, due to the reversible nature of both reactions, complete epimerization still occurs when the overall cascade converges towards the thermodynamic equilibrium for maximal conversions. If the follow-up reaction is rendered irreversible (e.g., *via* selective decarboxylation), a Curtin-Hammett type system is obtained, in which epimerization no longer occurs.[10d] Follow-
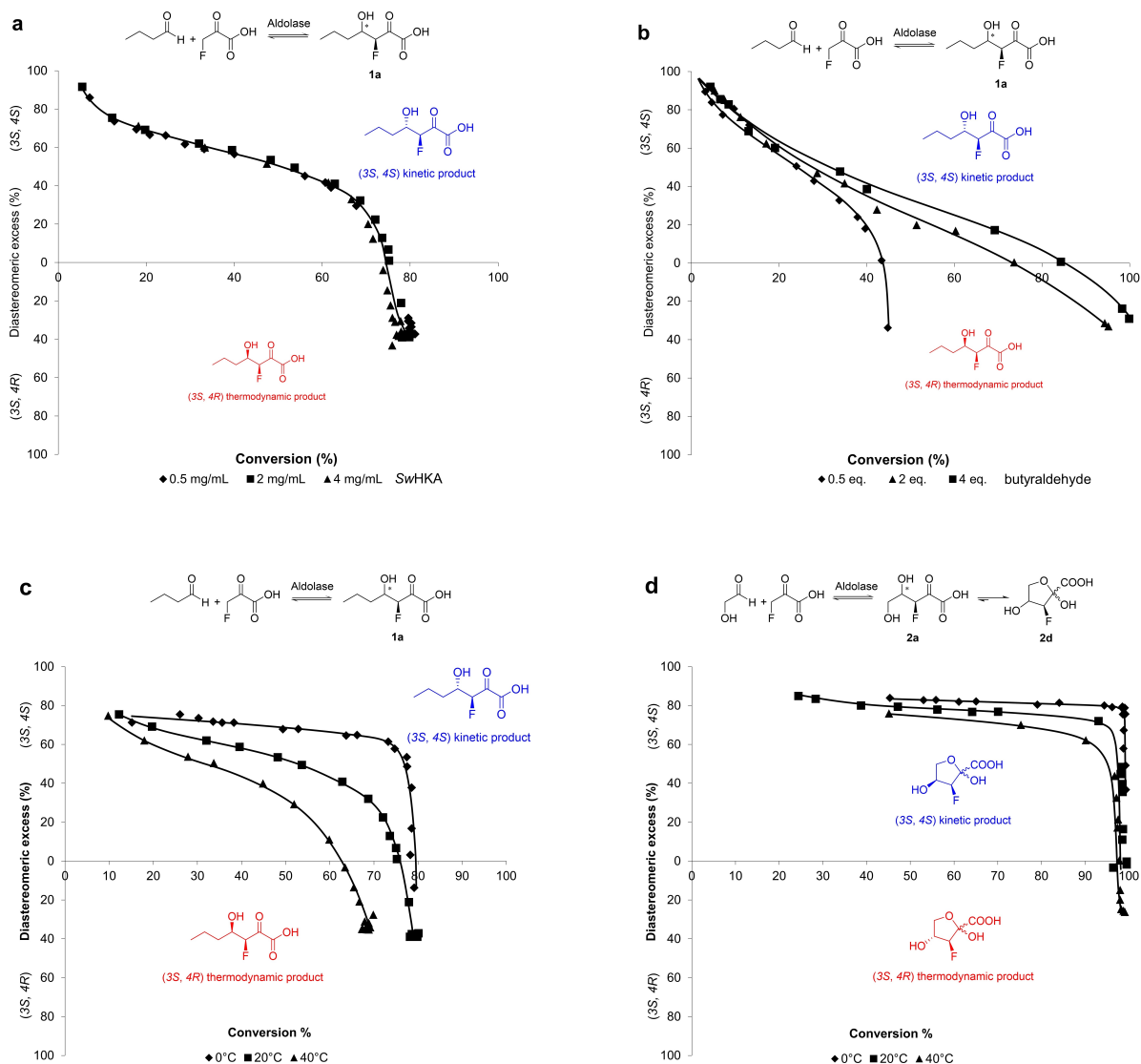
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Figure 4. SwHKA catalysed aldol reaction between fluoropyruvate and butyraldehyde (a–c) or fluoropyruvate and glycolaldehyde (d). a) The diastereomeric excess correlates with the extent of conversion and is independent of the catalyst loading. b) The use of one substrate in excess (here: butyraldehyde) allows for higher conversions and increases selectivity for the kinetic product. c) Temperature: SwHKA catalysed synthesis of **1a**. At 0 °C, the reverse reaction in the retro-aldol direction becomes kinetically negligible, which circumvents product epimerization and allows for higher conversions in the case of exergonic reactions. Conversely, elevated temperatures promote epimerization via higher rates in the retro-aldol direction. d) Follow-up reactions (here: ketal formation) increase the overall extent of conversion. This prolongs the duration of kinetically controlled conditions and allows for higher yields of the kinetic product.

up reactions are therefore a suitable means to increase diastereoselectivity for the kinetic product, and might even be used to render the reaction irreversible in some specific cases.[10d]

Quantum mechanical energy calculations

Given the evidence presented, we conclude that thermodynamic contributions can substantially reduce the diastereomeric excess of catalytic reactions and may even lead to the inversion of a stereocentre. Accurate thermodynamic predictions could enable rational choices between thermodynamically or kinetically controlled conditions at an early stage of process design.

The relative energies of formation for each diastereomer can be predicted by quantum mechanical (QM) modelling.

To demonstrate this approach, the lowest energy conformations of aldol products **1a** were found through a comprehensive conformational search with DFT. For the resulting conformers, the Gibbs energy of formation was predicted for an aqueous environment using a highly accurate complete basis set method. The computational results correctly predicted the (3S,4R) configuration of the thermodynamic product and were in good agreement with the experimentally observed diastereomeric excess ($de_{exp} = 37\%$, $de_{calc} = 55\%$, Figure 2a, Figure S8).

When considering industrial applications, the two-step synthesis of **2d** constitutes an excellent model system for the study of epimerization effects in sequential equilibrium reactions,

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such as organocatalytic cascade reactions,[17] cell-factories[18] and *in vitro* systems biocatalysis[19] (Figure 5). In this system, *Sw*HKA or *Bp*HKA catalyses the aldol reaction between fluoropyruvate and glycolaldehyde to yield the *syn* and *anti* configured diastereomers of **2a**. Spontaneous cyclisation subsequently affords both (2*R*) and (2*S*) configured anomers of each diastereomer. Following a similar approach, the free energies of formation were predicted for all diastereomers of **2a** and **2d**. Notably, (3*S*,4*S*) and (3*S*,4*R*) configured **2a** were predicted to afford a diastereomeric excess of only 2% under thermodynamic conditions. However, subsequent anomerization of **2a** affords (2*R*,3*S*,4*R*) configured **2d** as the overall thermodynamic product, and the predicted diastereomeric excess of (3*S*,4*S*) and (3*S*,4*R*) configured anomers was in excellent agreement with experimental results ($de_{exp} = 25\%$, $de_{calc} = 21\%$, Figure 2b, Figure S9).

The absolute configuration of the diastereomer that is overall lowest in energy determines the identity of the final product in sequential reactions, which may be opposite to the diastereoselectivity of catalysts that were used for the preceding reaction steps. Mechanistically reversible cascade reactions therefore converge toward the global, thermodynamic product; counteracting both the stereoselectivity of the used catalysts and the relative energies of all reaction intermediates.
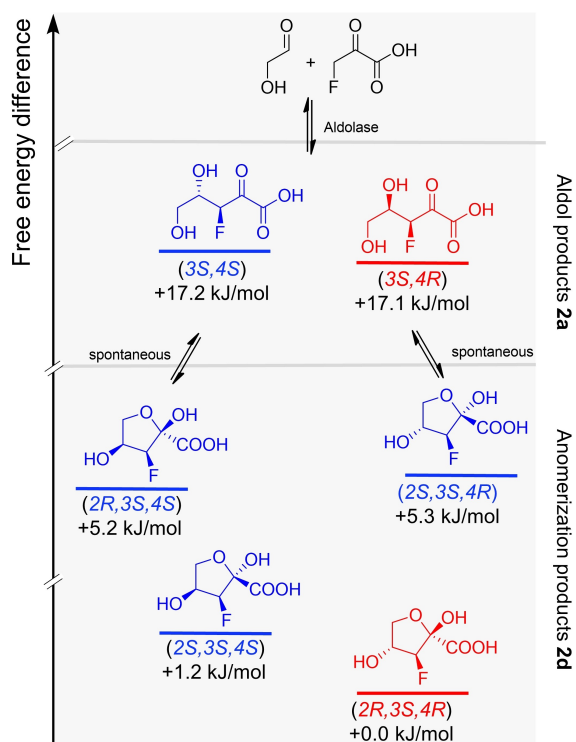
The reported error for the quantum chemical prediction of absolute Gibbs energies of formation by the CBS-QB3 method is approximately 4.6 kJ/mol.[20] Since epimers only differ in their absolute configuration at one stereocentre, systematic errors are largely cancelled out in the prediction of *relative* energy differences. In our case, an error of 0.2–0.8 kJ/mol (corresponding to ±4–16% diastereomeric excess) was observed with respect to experimental values of **1a** and **2d**. It remains to be established whether the low prediction errors are typical for this kind of calculations. The results do indicate that CBS-QB3, and QM methods that provide similar accuracy, are promising tools for providing predictions of the diastereomeric excess under equilibrium conditions.

Conclusion

A defining property of catalysts is the increase in rate, by which a reaction converges toward the thermodynamic equilibrium. In agreement with this notion, our results showed that catalysts simultaneously also accelerate the rate of epimerization during the synthesis of diastereomers. This effect can even lead to the inversion of a chiral centre against the catalyst's diastereoselectivity. The common practice of running a reaction to completion and subsequently deducing the catalyst's diastereoselectivity based on the absolute configuration of the isolated product therefore constitutes a concerning pitfall for catalysis. Predictions of the free energy difference between diastereomers could allow for a rational choice between thermodynamically or kinetically controlled conditions at an early stage of process design. Quantum mechanical calculations, with CBS-QB3, were indeed shown to accurately predict the relative energy difference between the investigated epimers. Kinetically controlled conditions were successfully established by reducing the temperature, but came at the expense of a lower rate of reaction. Notably, a similar effect was achieved by adding one substrate in excess. This did not only increase the extent of conversion, but also improved the selectivity toward formation of the kinetic product. While artificial cascade reactions promise a more sustainable, one-pot synthesis of stereocomplex molecules, the reversible nature of most organocatalytic- or enzymatic reactions inevitably introduces epimerization effects that need to be considered.

Figure 5. Sequential equilibrium reactions in a two-step cascade system. The aldolase catalysed coupling of fluoropyruvate and glycolaldehyde affords *syn* and *anti* configured **2a**, which spontaneously cyclise to afford the anomerization products **2d**. While **2a** constitutes a nearly racemic product mixture at C-4, extension of the reaction sequence by subsequent cyclisation yields the anomers of (3*R*,4*R*) configured **2d** as the overall thermodynamic product with a diastereomeric excess of 25%.

Experimental Section

Enzyme expression and purification: Detailed protocols next to gene and protein sequences are provided in the supporting information.

Retro-aldol activity assay: Purified *Sw*HKA or *Bp*HKA was incubated with NADH (0.5 mM), MgCl₂ (2 mM), L-lactic dehydrogenase (10 U/mL) in potassium phosphate buffer (5 mM, pH 7.5) and the reaction was initiated by the addition of oxaloacetate (0.5 mM) to afford a final volume of 1 mL. The change in absorbance was followed at 340 nm in PMMA cuvettes (800 rpm, 25 °C) in triplicate, using a Cary 60 UV-Vis spectrometer (Agilent Technologies) equipped with a TC1 stirring unit (Quantum Northwest).

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General protocol for aldol reactions to afford 1 a–3 a: Purified apo-*Sw*HKA or apo-*Bp*HKA (0.5–4 mg/mL) was incubated with $MgCl_2$ (2 mM, 2 minutes, room temperature) to afford the holoenzyme. The aldol reaction was initiated by the addition of sodium fluoropyruvate (50 mM) and the corresponding aldehyde (55 mM) to afford a final volume of 10 mL (5 mM potassium phosphate, 100 mM triethanolamine, pH 7.5, T = on ice, 20 °C and 40 °C, in a 10 mL glass screw cap vial, equipped with a stirring bar). Samples were taken over time to monitor the reaction by ^{19}F-NMR. Decarboxylation with H_2O_2 was used to analyse both the extent of conversion and the diastereomeric excess (Figure S6), while asymmetric reduction with L-lactic dehydrogenase was used to determine the absolute configurations (Figure S7). Equilibrium conditions were confirmed by the addition of fresh enzyme; no change was observed.

Decarboxylation of the ketoacid aldol products to afford 1 b–3 b: Samples of the crude reaction mixture (400 μL) were quenched by the addition of H_2O_2 (1% *wt/v*) and D_2O (10% *v/v*) and were incubated for 30 minutes at room temperature to decarboxylate the ketoacid aldol product. Residual H_2O_2 was quenched by the addition of catalase (0.02 mg/mL, 30 minutes, room temperature). KF was added as an internal standard (2 mM, $\delta = -120.0$ ppm) to afford a final volume of 500 μL. Precipitated protein was removed by centrifugation (10'000 rpm, benchtop centrifuge, 5 minutes) and the samples were analysed by ^{19}F-NMR. At low levels of conversion, spectra in which the signal-to-noise ratio of the minor product peak was below 2:1 were discarded, in order to reliably determine the diastereomeric excess in all cases. The diastereomeric excess was calculated from the integrals of the peaks corresponding to the (3S,4S) and (3S,4R) configured products. Conversions were determined with respect to the consumption of fluoropyruvate.

Reduction of the ketoacid aldol products to afford 1 c–3 c: Samples of the crude reaction mixture (400 μL) were mixed with EDTA (50 mM) and incubated for 30 minutes at room temperature. Inactivation of aldolase activity by EDTA under given conditions was demonstrated by the retro-aldol activity assay for the decarboxylation of oxaloacetate. L-lactic dehydrogenase (0.3 mg/mL) was added, using either stoichiometric amounts of NADH (65 mM, 2.0 eq.) or a glucose dehydrogenase (GDH) regeneration system (0.5 mM NADH, 200 mM D-glucose, 0.14 mg/mL GDH) for the asymmetric reduction of the aldol product (overnight, room temperature), after which D_2O (10% v/v) and KF (2 mM) were added to afford a final volume of 600 μL. Precipitated protein was removed by centrifugation (10000 rpm, 5 minutes) and the samples were analysed by ^{19}F-NMR.

Quantum mechanical modelling: The relative stability of each enantiomer was predicted using quantum mechanical calculations. Since the lowest energy conformations of the complex molecules could not be predicted by intuition, a comprehensive set of conformers was initially generated using standard dihedral angles. The geometries of the obtained molecules were subsequently optimised to find the lowest energy conformation of each enantiomer with quantum mechanical energy minimisations. The predicted Gibbs energies were then used to calculate the stability differences between the respective diastereomers. A more detailed protocol is provided in the supporting information and all scripts are available upon request.

Conflict of Interest

The authors declare no conflict of interest.

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Kinetic control: The free energy difference between two epimers determines the diastereomeric excess under equilibrium conditions. Due to the mechanistic reversibility and non-ideal stereoselectivity of catalysts, thermodynamic epimerization can occur against the catalyst's intrinsic stereoselectivity. This has far-reaching consequences for the characterisation of catalysts and their application in multi-step cascade reactions.
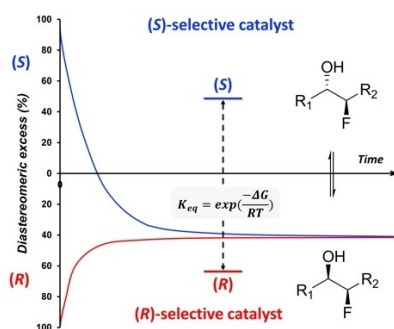
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Thermodynamics Determine the Diastereochemical Outcome of Catalytic Reactions