



## Size as a parameter for solvent effects on *Candida antarctica* lipase B enantioselectivity

Jenny Ottosson <sup>a</sup>, Linda Fransson <sup>a</sup>, Jerry W. King <sup>b</sup>, Karl Hult <sup>a,\*</sup>

<sup>a</sup> Department of Biotechnology, Royal Institute of Technology, Stockholm Center for Physics Astronomy and Biotechnology, SE-106 91 Stockholm, Sweden

<sup>b</sup> National Center for Agricultural Utilization Research, ARS/USDA, 1815 North University Street, Peoria, IL 61604, USA

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### Abstract

Changes in solvent type were shown to yield significant improvement of enzyme enantioselectivity. The resolution of 3-methyl-2-butanol catalyzed by *Candida antarctica* lipase B, CALB, was studied in eight liquid organic solvents and supercritical carbon dioxide, SCCO<sub>2</sub>. Studies of the temperature dependence of the enantiomeric ratio allowed determination of the enthalpic ( $\Delta_{R-S} \Delta H^\ddagger$ ) as well as the entropic ( $\Delta_{R-S} \Delta S^\ddagger$ ) contribution to the overall enantioselectivity ( $\Delta_{R-S} \Delta G^\ddagger = -RT \ln E$ ). A correlation of the enantiomeric ratio,  $E$ , to the van der Waals volume of the solvent molecules was observed and suggested as one of the parameters that govern solvent effects on enzyme catalysis. An enthalpy–entropy compensation relationship was indicated between the studied liquid solvents. The enzymatic mechanism must be of a somewhat different nature in SCCO<sub>2</sub>, as this reaction in this medium did not follow the enthalpy–entropy compensation relation. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** Enantiomeric ratio; Enthalpy; Entropy; Lipase; Resolution

### 1. Introduction

The use of enzymes as catalysts in organic chemistry is, although met with skepticism at first, a well-established and versatile technique [1,2]. Many enzymes, i.e. lipases, are surprisingly stable and active in organic solvents, retaining properties such as selectivity and activity under relatively mild reaction conditions, and provide a pleasing alternative to classical chemistry [3,4]. Enantioselectivity is an especially useful property of enzymatic action, enabling production of enantiopure substances. The demand

for enantiopure compounds is expected to increase [5] particularly within the pharmaceutical industry, as legislation now requires investigations on the pharmacological effects of each enantiomer of a chiral drug [6,7]. Furthermore, enantioselectivity serves as an excellent model system for research on the mechanisms of enzyme selectivity as the substrates have identical ground state energies. It has been shown many times that enzymes are strongly affected by the choice of solvent they are used in. As a matter of fact even reversal of substrate specificity [8,9] and enantiopreference [10,11] due to solvent changes have been observed. Solvent engineering to optimize enzyme selectivity, i.e. enantioselectivity, for target reactions would be a very appealing and efficient alternative to protein engineering [2]. Consequently many

\* Corresponding author. Fax: +46-8-5537-8468.  
E-mail address: kalle@biochem.kth.se (K. Hult).

researchers have put considerable effort into elucidating the underlying mechanisms responsible for the observed solvent effects. Several models have been proposed, which have been successful for particular enzymatic systems, but none so far has shown general applicability.

One such model is based on the hypothesis that solvent effects are due to the differential free energy of desolvation of the substrates in their enzyme-bound transition states. The methodology involves molecular modeling calculation of the desolvated part of the substrates and the estimation of their thermodynamic activity coefficients,  $\gamma$ , using the UNIFAC computer algorithm [12]. In this model, the logarithm of enzyme selectivity is proposed to show a linear dependence to the logarithm of the ratio of the substrates activity coefficients. In the case of enantiomeric substrates:  $\log E = \log(\gamma_R/\gamma_S) + \text{constant}$ . Kinetic resolutions catalyzed by cross-linked crystals of  $\gamma$ -chymotrypsin and prochiral selective catalysis with cross-linked crystals of  $\gamma$ -chymotrypsin and subtilisin Carlsberg are examples of systems that have been described by this model [13,14]. However the model did not hold for solvent effects on enzyme selectivity in predominantly aqueous media [15], nor did it hold for some resolutions catalyzed by lyophilized or cross-linked crystals of subtilisin [16]. In the cases where the model does not hold other effects apart from substrate desolvation must come into play, such as solvent displacement or conformational changes on the enzyme.

Several solvent characteristics have been shown to affect the success of enantioselective enzyme reactions and attempts to find correlations to the enantiomeric ratio,  $E$ , have been made. The most studied relation is the relation between  $E$  and solvent hydrophobicity, measured as  $\log P$  where  $P$  is the partition coefficient of the solvent between octanol and water. Although several studies indicate a decrease in  $E$  with increasing  $\log P$  [11,17–21] this is by no means a general observation. Exceptions include complete lack of correlation [22], bell-shaped relations [23,24] and increasing  $E$  with increasing  $\log P$  [25] that also have been observed. In addition to this, solvent characteristics such as dipole moment and dielectricity constant [25] have been found in some systems to provide a correlation to  $E$ .

In the present investigation a thermodynamic anal-

ysis of the enantioselectivity of *Candida antarctica* lipase B, CALB, in nine different solvents is presented. This type of analysis provides the additional information of differential activation enthalpy and entropy and their changes in different solvents, which is not available when studying only the variation in the enantiomeric ratio. A correlation between the size of the solvent molecule and  $E$  is suggested. A better understanding of the mechanisms of solvent effects on enzyme catalysis would allow for more rational experimental design relieving the resources nowadays spent on screening and protein engineering for an optimal biocatalyst. This development could enable simple solvent changes to be sufficient to acquire the required enzyme selectivity.

### 1.1. Theory

The enantiomeric ratio  $E$ , defined as the ratio between the specificity constants for the competing enantiomeric substrates by Chen et al. [26], is related to the difference in activation free energy between the enantiomers as  $\Delta_{R-S}\Delta G^\ddagger = -RT\ln E$  in an enantioselective enzyme-catalyzed reaction. Furthermore,  $\Delta_{R-S}\Delta G^\ddagger$  is related to the difference in activation enthalpy and entropy as  $\Delta_{R-S}\Delta G^\ddagger = \Delta_{R-S}\Delta H^\ddagger - T\Delta_{R-S}\Delta S^\ddagger$ . The enthalpic and entropic component of the enantiomeric ratio  $E$  is equated according to Eq. 1 [27,28].

$$\ln E = -\frac{\Delta_{R-S}\Delta H^\ddagger}{R} \cdot \frac{1}{T} + \frac{\Delta_{R-S}\Delta S^\ddagger}{R} \quad (1)$$

Hence, studies on the temperature dependence of  $E$  allows for a thermodynamic analysis of enantioselectivity. Enthalpic differences between the enantiomers during catalysis are easy to visualize as the enantiomer's steric differences during catalysis. For *C. antarctica* lipase B, CALB, these steric differences in catalysis of secondary alcohols have been elucidated through a combination of experimental and computer modeling research [29]. The entropic activation energy differences of enantiomeric substrates can however not as clearly be accounted for on a molecular level. Nevertheless, it has been shown that both enthalpic and entropic differences are significant to enzyme enantioselectivity [27,28,30–34]. In the present investigation a thermodynamic analysis of the effect of different solvents on the enantioselectivity

tivity of the transesterification of 3-methyl-2-butanol catalyzed by *C. antarctica* lipase B, CALB, is presented (Scheme 1). The enzyme prefers the *R*-enantiomer of this chiral substrate and previous studies have shown that the *R*-enantiomer is enthalpically favored ( $\Delta_{R-S}\Delta H^\ddagger$  is negative) but entropically unfavored ( $\Delta_{R-S}\Delta S^\ddagger$  is negative) [31]. In other words the entropic component counteracts the enthalpic one, yielding a lower enantioselectivity than expected from purely enthalpic considerations. This is true at experimental temperatures below the racemic temperature,  $T_r = \Delta_{R-S}\Delta H^\ddagger / \Delta_{R-S}\Delta S^\ddagger$ , as in the present study. At  $T_r$  the enantioselectivity is zero and above it the selectivity changes to the entropically preferred enantiomer and enantioselectivity for that enantiomer will increase with temperature.

## 2. Materials and methods

Kinetic resolution of 3-methyl-2-butanol through transesterification catalyzed by *C. antarctica* lipase B was studied in eight organic solvents and supercritical CO<sub>2</sub>, SCCO<sub>2</sub>, Scheme 1. Equilibration of the enzyme preparation, Novozyme 435, to a water activity of 0.1 (LiCl (aq., sat.)) and drying of all reagents over molecular sieves ensured a low water content in the system, preventing hydrolytic side reactions.

### 2.1. Reactions in liquid solvent

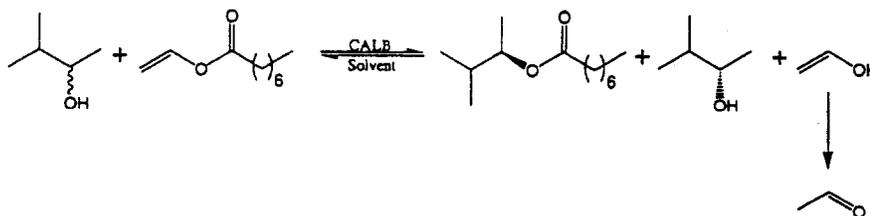
Enzyme preparation, 10–305 mg, 3-methyl-2-butanol (0.43 M) and solvent was allowed to equilibrate in temperature. Addition of the acyl donor vinyl octanoate (0.43 M) in a total volume of 2.3 or 5 ml started the reaction. Samples were taken at regular intervals between 0 and 50% conversion.

### 2.2. Reactions in SCCO<sub>2</sub>

The enzymatic reactions were carried out in a Spe-ed unit from Applied Separations modified as depicted in Fig. 1A, with a gage snubber from Chemiquip Products Co., rebuilt to function as a high pressure stirred reactor assembly with a volume of 5 ml (Fig. 1B). Enzyme, 50 mg, and 420  $\mu$ l vinyl octanoate ( $\approx 0.43$  M) was placed in the reaction chamber. The reaction chamber was then installed in the set-up, pressurized and left to equilibrate (all valves closed) in temperature and pressure (13.8 MPa) for more than 4 h, usually more than 10. A Rheodyne Model 7125 Syringe Loading Sample Injector enabled the injection of 234  $\mu$ l 3-methyl-2-butanol ( $\approx 0.43$  M) to start the reaction by final pressure increase to 21.4 MPa. At this pressure and 40°C carbon dioxide has the same solubility parameter as hexane (7.3 (cal/ml)<sup>1/2</sup>). Samples were collected at regular intervals using the four top valves. These valves enabled sampling followed by rinsing of the tubing with hexane to avoid sample-to-sample carry over, flushing the tubing with CO<sub>2</sub> and then re-pressuring of the reaction chamber with CO<sub>2</sub>.

### 2.3. Sample analysis and determination of the enantiomeric ratio

Sample analysis on chiral capillary GC enabled determination of the enantiomeric excess of the remaining substrate 3-methyl-2-butanol,  $ee_s$ , and the produced octanoate ester,  $ee_p$ . The column used was a Chirasil-Dex CB from Chrompack, the Netherlands with which baseline separation of the enantiomers was achieved in less than 15 min. For both the remaining substrate and the product the enantiomer in minor concentration was first in the elution order. The enantiomeric ratio,  $E$ , was calculated as



Scheme 1. Kinetic resolution of 3-methyl-2-butanol catalyzed by *Candida antarctica* lipase B, CALB.

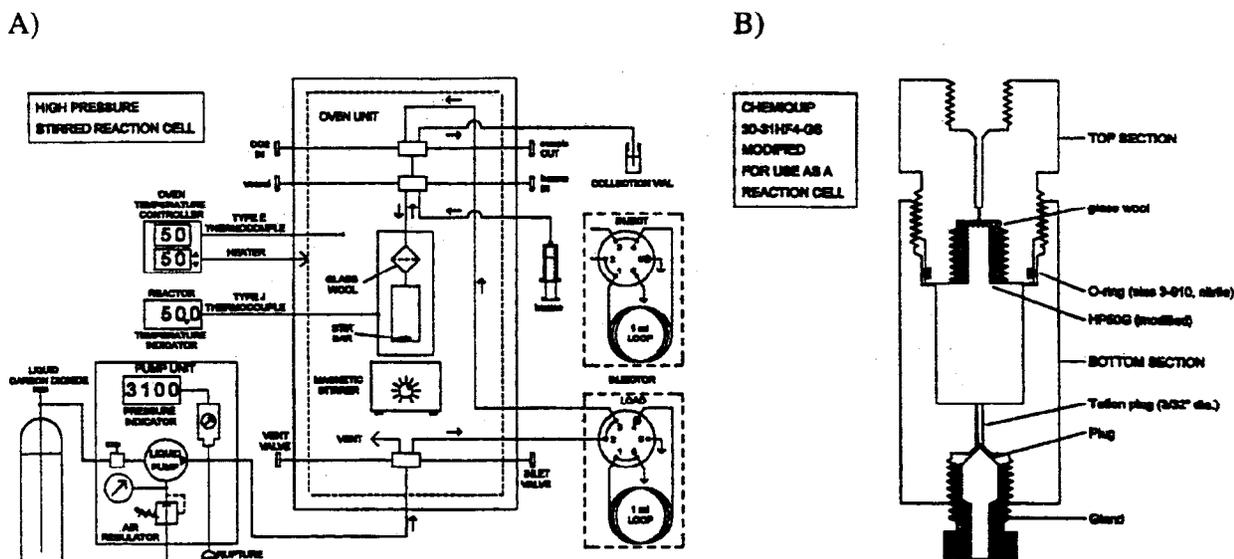


Fig. 1. (A) Experimental setup and (B) reaction chamber for the resolution of 3-methyl-2-butanol catalyzed by *C. antarctica* lipase B in supercritical carbon dioxide SCCO<sub>2</sub>.

an average of 4–14 samples at conversions 0–50% according to Rakels et al. [35].

### 3. Results

In the present study it was found that the success of the kinetic resolution of 3-methyl-2-butanol catalyzed by *C. antarctica* lipase B, CALB, was highly

dependent on the solvent. The best enantioseparation was achieved in the molecularly larger hydrophobic solvents *cis*-decaline and *n*-hexane, whereas supercritical carbon dioxide, SCCO<sub>2</sub>, displayed the lowest degree of enantioselectivity, (Fig. 2). The other six liquid solvents had intermediate enantiomeric ratios, *E*. *E* was also determined in hexane under pressure (21.4 MPa) and showed no significant change compared to the value for the reaction in hexane at at-

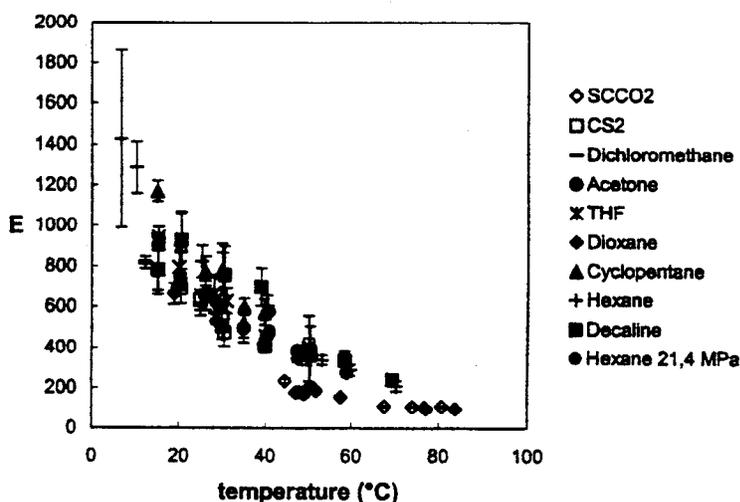


Fig. 2. Enantiomeric ratio as a function of temperature in the kinetic resolution of 3-methyl-2-butanol in organic solvents and supercritical CO<sub>2</sub>, SCCO<sub>2</sub>. Error bars display  $\pm$  one standard deviation.

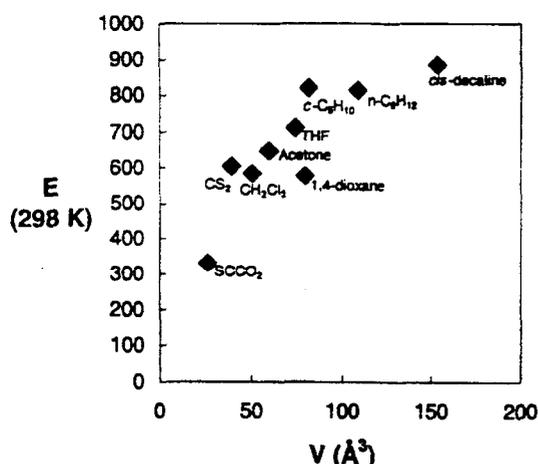


Fig. 3. Enantiomeric ratio at 298 K as a function of molecular volume of solvent. In the case of  $\text{SCCO}_2$  the value is extrapolated out of the experimental temperature range with the linear relation of  $\ln E$  vs.  $1/T$ .

mospheric pressure (Fig. 2). A complete overlap of the experimental temperature ranges for the reaction systems was not possible due to the different boiling points of the solvents and the critical temperature of  $\text{CO}_2$ . However by extrapolation of Eq. 1 out of the experimental temperature range, an  $E$  at 298 K was determined for  $\text{SCCO}_2$ . In Fig. 3 the enantiomeric ratios at 298 K for each reaction system are plotted as a function of the solvent's van der Waals volume. This plot indicates a correlation between these parameters, the larger the solvent the larger the enantioselectivity and vice versa. Table 1 presents the differential activation enthalpy,  $\Delta_{R-S}\Delta H^\ddagger$ , and entropy,  $\Delta_{R-S}\Delta S^\ddagger$ , terms of enantioselectivity determined

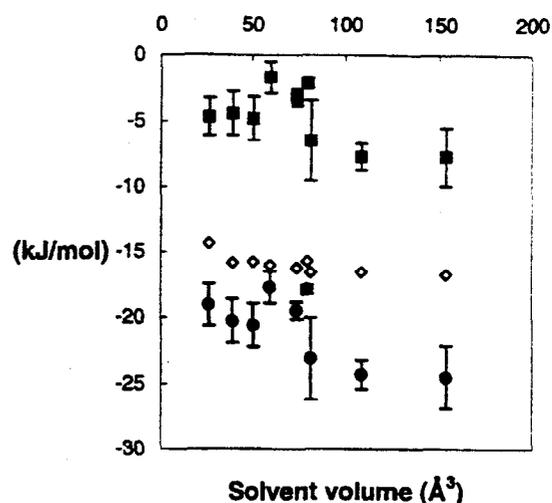


Fig. 4. Differential activation free energy ( $\diamond$ ),  $\Delta_{R-S}\Delta G^\ddagger$ , and its enthalpic ( $\bullet$ ),  $\Delta_{R-S}\Delta H^\ddagger$ , and entropic ( $\blacksquare$ ),  $T\Delta_{R-S}\Delta S^\ddagger$ , components at 298 K for the enantioselective transesterification of 3-methyl-2-butanol catalyzed by *C. antarctica* lipase B in solvents of different molecular volumes. Error bars are calculated from the standard errors of the linear regression of  $\ln E$  vs.  $1/T$ .

from the temperature dependence of  $E$ . The temperature dependence of *cis*-decaline and *n*-hexane were nearly identical and consequently, their  $\Delta_{R-S}\Delta H^\ddagger$  and  $\Delta_{R-S}\Delta S^\ddagger$  were so as well. One notes that the observed changes in  $E$  with solvent were caused both by changes in the enthalpic as well as the entropic term,  $\Delta_{R-S}\Delta H^\ddagger$  and  $\Delta_{R-S}\Delta S^\ddagger$ . Fig. 4 presents the individual components,  $\Delta_{R-S}\Delta H^\ddagger$  and  $\Delta_{R-S}\Delta S^\ddagger$ , as well as the combined activation free energy difference between the enantiomers,  $\Delta_{R-S}\Delta G^\ddagger$ , as a function of solvent size. A stepwise change of the indi-

Table 1

Thermodynamic components of the enantiomeric ratio,  $E$ , for *Candida antarctica* lipase B transesterification of 3-methyl-2-butanol in non-aqueous solvents

Solvent	Volume ( $\text{\AA}^3$ )	$E$ (298 K)	$\Delta\Delta G^\ddagger$ (298 K) (kJ/mol)	$\Delta\Delta S^\ddagger$ (J/mol, K)	$\Delta\Delta H^\ddagger$ (kJ/mol)	$T_r$ (K)
Decaline	154	890	-16.8	$-26 \pm 7.5$	$-25 \pm 2.4$	950
Hexane	109	810	-16.6	$-26 \pm 3.4$	$-24 \pm 1.1$	940
Cyclopentane	82	820	-16.6	$-22 \pm 10.2$	$-23 \pm 3.1$	1060
1,4-Dioxane	80	580	-15.8	$-7.3 \pm 1.1$	$-18 \pm 0.3$	2460
Tetrahydrofuran	74	710	-16.3	$-11 \pm 2.1$	$-20 \pm 0.6$	1790
Acetone	60	650	-16.0	$-5.7 \pm 4.0$	$-18 \pm 1.2$	3090
Dichloromethane	50	580	-15.8	$-16 \pm 5.5$	$-21 \pm 1.6$	1270
Carbon disulfide	39	600	-15.9	$-15 \pm 5.6$	$-20 \pm 1.7$	1370
$\text{SCCO}_2$	26	330 <sup>a</sup>	-14.4 <sup>a</sup>	$-16 \pm 4.7$	$-19 \pm 1.6$	1210

Errors are  $\pm$  standard error of the linear regression of  $\ln E$  vs.  $1/T$ .  $E$  at 298 K was calculated from the linear relation.

<sup>a</sup>Value calculated with extrapolation out of the experimental temperature range.

vidual components was noted that could not be discerned in the overall  $\Delta_{R-S}\Delta G^\ddagger$ . The smallest solvents (SCCO<sub>2</sub>, CS<sub>2</sub> and CH<sub>2</sub>Cl<sub>2</sub>) had intermediate absolute values of  $\Delta_{R-S}\Delta S^\ddagger$  and  $\Delta_{R-S}\Delta H^\ddagger$ , the medium-sized solvents (acetone, THF and 1,4-dioxane) showed low values and the largest solvents (*cyclo*-pentane, *n*-hexane and *cis*-decaline) had the largest values. Furthermore, the racemic temperature,  $T_r$ , was well above (> 660 K) the experimental temperature range for all the reaction systems studied and consequently  $E$  decreases with temperature (Table 1).

#### 4. Discussion

Solvent engineering has great potential for the use of enzymes in organic chemistry. Improvement of enzyme enantioselectivity through rational choice of solvent could relieve the need for time and resource consuming screening or protein engineering of the biocatalyst [2]. As described in Section 1, much effort have been spent at trying to elucidate the underlying mechanism of solvent effects to be able to predict them to allow such rational solvent engineering. From the present investigation (Fig. 3) we conclude that the size of the solvent molecules may be one of the parameters that governs the success of enzymatic resolution of a chiral reagent.

A combined effort with molecular modeling and experimental research led Orrenius et al. to propose a model of how *C. antarctica* lipase B, CALB, distinguishes between the enantiomers of *sec*-alcohols [29]. This model involves two different modes of

binding for the enantiomers in order to develop the transition state hydrogen-bonding pattern required for catalysis, Fig. 5. The steric difference between the enantiomers in their respective transition state is the origin of the enzymes ability to discriminate between the enantiomers according to the model. However, as seen in this and several other studies, enantioselectivity of enzymes is not only caused by enthalpic activation energy differences of the enantiomers, as suggested by the model, but also entropic,  $\Delta_{R-S}\Delta S^\ddagger$  [27,28,30–34]. The fact that the model involves different orientations of the enantiomers in transition state, means that the activated complex will be solvated differently in the enzyme during catalysis. Consequently, the interactions with solvent molecules will be quite different for the enantiomers in transition state. The difference in activation entropy between the enantiomers must be caused by different transition state entropy of the substrate or protein, but can in part also be the result of different displacement of solvent from the active site. The number of solvent molecules involved in the solvation of the active site may be different for the enantiomers. A consequence of this would be that the entropic activation energy difference should depend on the size of the solvent molecules as observed. A large solvent will loose translational entropy of fewer solvent molecules than a smaller solvent. In hexane and decaline, the thermodynamic analysis of  $E$  was practically identical. This suggests an upper limit for the effects correlated to solvent volume by which the solvents are so large that there is no difference in the number of solvating molecules between the enan-

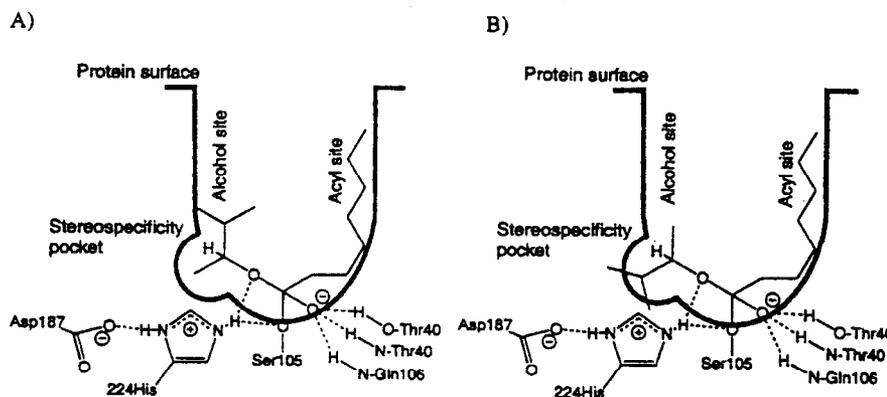


Fig. 5. Cartoon of the proposed transition state binding modes of *C. antarctica* lipase B [29]. (A) The preferred enantiomer (*R*)-3-methyl-2-butanol; (B) the non-preferred enantiomer (*S*)-3-methyl-2-butanol.

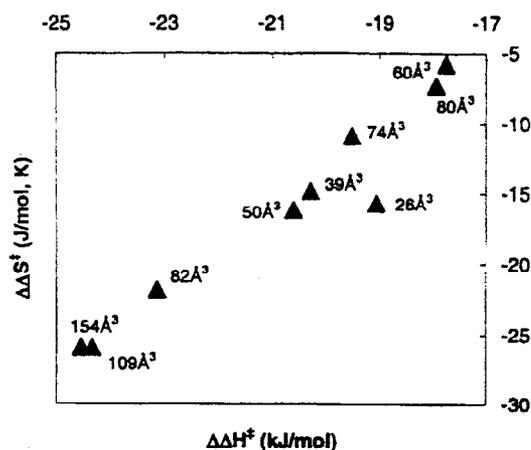


Fig. 6. Enthalpy–entropy compensation plot of the kinetic resolution of 3-methyl-2-butanol catalyzed by *C. antarctica* lipase B.  $R^2$  from a linear regression is 0.9904 if  $\text{SCCO}_2$  ( $26 \text{ \AA}^3$ ) is excluded and 0.9367 when included.

tiomers. The remaining  $\Delta_{R-S}\Delta S^\ddagger$  has other origins such as difference in entropy of the substrate itself or freedom of certain enzyme residues between the enantiomer's transition states.

As mentioned in the introduction a lot of effort has been made to correlate  $E$  to different solvent characteristics in the quest for a simple correlation that would allow for rational solvent engineering. In this investigation no correlation was found to the solvents' dielectricity constants. However, somewhat of a correlation of  $E$  to  $\log P$ , a common measure of solvent hydrophobicity, was observed. The largest  $E$  was found in the solvents of highest  $\log P$  and vice versa. However, there is also a correlation between the solvents molecular volume and their hydrophobicity,  $\log P$ . So whether the observed changes are actually related to changes in  $\log P$  or molecular volume we cannot with confidence say until a large solvent of low  $\log P$  or a small solvent of high  $\log P$  have been tried. Such solvents are not easily thought of. However, the recent finding that enzyme catalyzed reactions function in ionic liquids may be the solution [36]. From this study and others in the literature, it is obvious that solvent effects on enzyme catalysis is a complex matter with several important parameters to include for a full model of the effects.

Fig. 6 displays the entropic component,  $\Delta_{R-S}\Delta S^\ddagger$ , of Eq. 1, as a function of the enthalpic component,  $\Delta_{R-S}\Delta H^\ddagger$ , with a strikingly good linear correlation. This type of enthalpy–entropy compensation (EEC)

phenomena has been observed many times in other reaction systems [31,37–41]. However, several authors have pointed out the dangers of drawing chemical interpretations from usually highly correlated plots of enthalpy versus entropy [42–47]. McBane illustrates this very clearly [42], with Arrhenius plots based on data randomly chosen from a faculty phone book that shows strikingly good enthalpy–entropy compensation, which of course is purely mathematical in nature without any chemical meaning. Although data may be insufficient to determine with certainty whether a real chemical compensation exists, one may note that the reaction catalyzed in supercritical  $\text{CO}_2$  ( $26 \text{ \AA}^3$ ) is an outlier in the EEC-plot (Fig. 6). This implies that the reaction in this solvent is different in mechanism compared to the reactions in the condensed media. This notion is strengthened when studying Fig. 7,  $\ln E = f(1/T)$ , in such a plot a real chemical compensation pattern of enthalpy and entropy should yield a single intersection for all reactions in the reaction series [42]. Though there is not an obvious common intersection for the liquid solvents in this plot, it is clear that the  $\text{SCCO}_2$  reaction is again an outlier.

Krug et al. set up a criterion to verify any real chemical compensation effect from artifactual statistical ones, in which the slope  $\beta$  of the EEC-plot is compared with the harmonic mean temperature  $T_{\text{HM}}$  of the data points. A sufficient criterion for the existence of a true chemical compensation is that  $\beta$ , also called the compensation temperature, is different from  $T_{\text{HM}}$ . In our case,  $T_{\text{HM}}$  was not con-

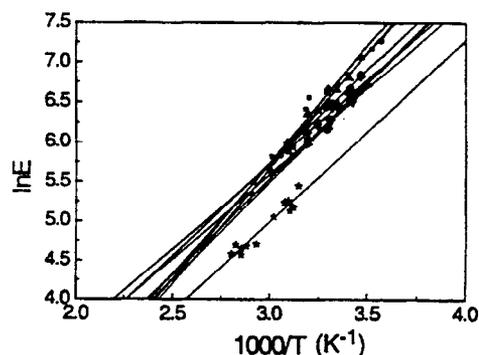


Fig. 7. Arrhenius plots of the enantioselective resolution of 3-methyl-2-butanol catalyzed by *C. antarctica* lipase B. Star,  $\text{SCCO}_2$ ; hexagon,  $\text{CS}_2$ ; triangle right,  $\text{CH}_2\text{Cl}_2$ ; triangle left, acetone; diamond, THF; triangle down, 1,4-dioxane; triangle up,  $n\text{-C}_5\text{H}_{10}$ ; circle,  $n\text{-C}_6\text{H}_{14}$ ; square, *cis*-decaline.

stant between the studied solvents, but varied between the data points in the region 298–333 K, with the median 304 K and a 95% confidence interval for the compensation temperature  $\beta$  given by (265–420) K. The harmonic mean temperature  $T_{HM}$  clearly fell inside the confidence interval and it is thus impossible to conclude a real chemical compensation effect at the 95% confidence level. The picture changed if SCCO<sub>2</sub>, which had been judged as an outlier, was excluded. Then the variation of  $T_{HM}$  was between 298 and 318 K with the median 304 K, and the 95% confidence interval for  $\beta$  was given by (309–371) K. Now most of the harmonic mean temperatures fell outside the confidence interval, indicating a higher likelihood of a true chemical compensation effect. One possibility to improve the statistics of enthalpy–entropy analysis is to increase the experimental temperature interval. For enzymatic systems this is generally an impossible solution since these systems are restricted by enzyme inactivation at high temperatures and low activity at low temperatures.

In what sense the enzyme mechanism is altered in the supercritical fluid remains for future research to elucidate. However, lysine residues on the protein surface can be covalently modified by CO<sub>2</sub> and could perhaps cause conformational changes resulting in a different mechanism [48]. Moreover, the presence of CO<sub>2</sub> may cause association with the bound water molecules on the enzyme, lowering the effective pH in the enzyme microenvironment, and thereby affect the catalysis [49]. It has been shown that enzyme conformation of crystals does not change significantly in organic and water media. If there are any conformational changes in CO<sub>2</sub> remains unclear [50,51]. Pressure alone, however, is not the cause of the different behavior of the reaction in SCCO<sub>2</sub>, as determination of  $E$  in hexane pressurized to 21.4 MPa showed no significant change in  $E$  compared to atmospheric pressure.

Solvent engineering of enzyme-catalyzed reactions shows great potential as a means to optimize the success of the reactions. However, solvent effects on enzyme reaction systems are complex due to a number of physical properties changed when going from one solvent to another. Still, considerable research is required before the underlying mechanisms of solvent effects are likely to be elucidated to the

extent of allowing for such rational experimental design. The effects are likely to be caused by several different and intercorrelated contributions to the reaction energetics. The present investigation leads us to suggest that one of these contributions is the size of the solvent molecule.

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