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Temperature effects on S_1- and S'_1-enantioselectivity of α-chymotrypsin

B. Galunsky *, S. Ignatova, V. Kasche

Department of Biotechnology II, Technical University of Hamburg-Harburg, D 21071 Hamburg, Germany

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Abstract

The temperature dependence of E (enantiomeric ratio or enantioselectivity, a quantitative measure for enzyme stereospecificity) has been studied for the α-chymotrypsin catalysed hydrolysis of the enantiomeric *N*-Boc-L/D-TyrOMe, L/D-TyrOMe, Ac-L/D-PhgOMe, L/D-PhgOMe and for the kinetically controlled synthesis of the diastereomeric dipeptides *N*-Ac-L-Tyr-L/D-$ArgNH_2$ and *N*-Ac-L-Tyr-L/D-$ValNH_2$. The results show that the S_1- and S'_1-enantioselectivity can be modulated by the temperature (3–15 fold for the studied substrates in the range 5–45°C). For L/D-PhgOMe a reversal in stereospecificity was found in this temperature interval. For the studied substrates both an increase or decrease of the enantiomeric ratio with increasing temperature was observed. For these processes the following relation for the temperature dependence of E has been derived $\ln E = \ln(k_L/k_D) = -(\Delta\Delta H^{\#} - \Delta\Delta H_b)/RT + (\Delta\Delta S^{\#} - \Delta\Delta S_b)/R$ where k_L and k_D are apparent second order rate constants for the reactions with the L- and D-enantiomers, respectively. $\Delta\Delta$ denotes the differences between the thermodynamic parameters for transformation of the enantiomeric substrates. The subscript $_b$ applies for the binding of the substrate or the nucleophile and the superscript $^{\#}$ for the formation of the transition state of the enzyme acylation or deacylation. For the studied processes either the enthalpy $(\Delta\Delta H^{\#} - \Delta\Delta H_b)$ or the entropy $(\Delta\Delta S^{\#} - \Delta\Delta S_b)$ term was found to control the discrimination. Thus, the enantioselectivity decreases or increases with temperature, respectively. The influence of ground-state interactions and transition-state stabilisation on enzyme enantioselectivity has been discussed. © 1997 Elsevier Science B.V.

Keywords: α-Chymotrypsin; Enantioselectivity; Enantioselectivity modulation

1. Introduction

Biochemical processes in vivo are inherently chiral [1,2] and most of the processes relevant to biological activity should be strongly dependent on the enantiomer purity of the chiral pharmaceuticals, agrochemicals and other biologically active substances [3,4]. Thus, the production of only one enantiomer of the above compounds will continue to be a general feature for the use of enzymes in synthetic organic chemistry and enzyme biotechnology [5–8]. Stereo(enantio)specificity of the enzymes is determined (as for the non enzymatic processes [9]) by the different stabilisation of the ground and the transition states of the enantiomeric substrates [10–12] (Fig. 1). This implies competition of enthalpy and entropy factors and can be influenced by the structure of the enzymes, substrates or the reaction conditions [13–17]. The temperature dependence of the enantiospecificity of several dehydrogenases and esterases has been studied in a few recent papers, which are the first to demonstrate temperature dependent stereo-

* Corresponding author. Fax: +49 40 77182127; E-mail: Galunsky@tu-harburg.d400.de

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chemical reversal of enzyme catalysed reactions [10,18–20].

A quantitative measure for the stereospecificity of an enzyme catalysed process is the ratio of the specificity constants for transformation of both enantiomers (enantioselectivity or enantiomeric ratio, $E = (k_{cat}/K_m)_L/(k_{cat}/K_m)_D$) [11,12]. For hydrolysis of α-amino acid esters or amides catalysed by proteinases, E reflects the S_1-enantioselectivity [21]. For α-chymotrypsin it has been shown that

$$\frac{k_{cat}}{K_m} = \frac{k_2}{K_S}, \quad (1)$$

where k_2 is the acylation rate constant and K_S is the true binding constant for the enzyme–substrate complex [22]. Thus, E is only influenced by differences in the binding and acylation reactions (Fig. 1(A)). Then the temperature dependence of enantioselectivity can be derived by the transition state theory and is given [23] by the relation

$$\ln E = -\frac{\Delta\Delta H}{RT} + \frac{\Delta\Delta S}{R} = -\frac{\left(\Delta\Delta H^{\#}_{ac} - \Delta\Delta H_b\right)}{RT} + \frac{\left(\Delta\Delta S^{\#}_{ac} - \Delta\Delta S_b\right)}{R}, \quad (2)$$

where $\Delta\Delta$ denotes the differences between the thermodynamic parameters for the reactions of the enantiomeric substrates, $_b$ applies for binding and $_{ac}^{\#}$ for transition state of enzyme acylation. For hydrolase-catalysed kinetically controlled synthesis of condensation products, the nucleophile or S'_1-enantioselectivity [21] can be determined by the ratio of the transferase to hydrolase ratios for the enantiomeric nucleophiles $E' = (k_T/k_H)_L/(k_T/k_H)_D$. The transferase to hydrolase ratio, assuming equilibrium in nucleophile binding, is given by the relation

$$\frac{k_T}{k_H} = \frac{k_d}{\left(k_h K_N + k_{h,N}[NH]\right)}, \quad (3)$$

where k_T/k_H is calculated from the initial rates of accumulation of the condensation and hydrolysis products, k_d is the deacylation rate constant of the acyl-enzyme–nucleophile complex by the bound nucleophile, k_h is the deacylation rate constant of the acyl-enzyme by water and $k_{h,N}$ is the deacylation rate constant of the acyl-enzyme–nucleophile complex by water [24]. When the above complex cannot be deacylated by water, transition state theory gives the following relation for E′

$$\ln E' = -\frac{\Delta\Delta H'}{RT} + \frac{\Delta\Delta S'}{R} = -\frac{\left(\Delta\Delta H^{\#}_d - \Delta\Delta H_{NH}\right)}{RT} + \frac{\left(\Delta\Delta S^{\#}_d - \Delta\Delta S_{NH}\right)}{R}, \quad (4)$$

where $\Delta\Delta$ have the same meaning as in Eq. (2), $_{NH}$ denotes nucleophile binding and $_d^{\#}$ transition state of acyl-enzyme deacylation by the bound nucleophile (Fig. 1(B)). When $\ln E(E')$ is a linear function of $1/T$, the temperature dependence of enthalpy and entropy differences in Eqs. (2) and (4) can be neglected. For L-specific enzymes the activation energy differences $(\Delta\Delta H - T\Delta\Delta S)$ in these equations are always < 0. An increase in $E(E')$ with temperature is only possible when $\Delta\Delta H(\Delta\Delta H')$ and $\Delta\Delta S(\Delta\Delta S')$

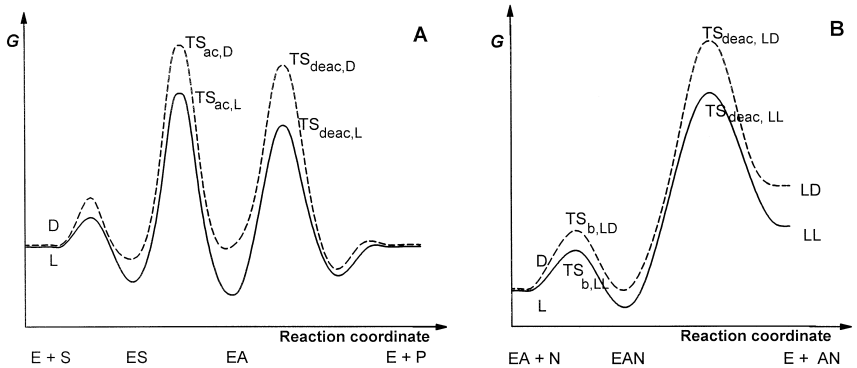

Fig. 1. Schematic free energy diagrams for the discrimination between the enantiomeric substrates in hydrolytic (A) and synthetic (B) reactions, catalysed by α-chymotrypsin. TS denotes transition state, subscripts $_{ac}$ and $_{deac}$ denote acylation and deacylation, respectively.

are >0. Using Eqs. (2) and (4) $\Delta\Delta H(\Delta\Delta H')$ and $\Delta\Delta S(\Delta\Delta S')$ can be determined from the temperature dependence of the stereoselectivity. The temperature at which the specificity constants for both enantiomers are equal (i.e. $E(E')=1$) is defined as ''reversal'' or ''racemic'' temperature [19]:

$$T_r = \frac{\Delta\Delta H}{\Delta\Delta S} \quad \text{or} \quad T_r = \frac{\Delta\Delta H'}{\Delta\Delta S'}. \tag{5}$$

When $T < T_r$, the discrimination is dominated by $\Delta\Delta H(\Delta\Delta H')$ and the stereochemical purity decreases with increasing temperature. The opposite is valid for $T > T_r$, where $T\Delta\Delta S(T\Delta\Delta S')$ controls the reaction.

The enantioselectivity of hydrolytic enzymes is important for racemate resolution and for stereospecific enzyme catalysed synthesis of condensation products (peptides, esters, β-lactam antibiotics etc.) [6,7,15,25,26]. Optimal enzymes for these processes should have $E > 100$ for L- or $E < 0.01$ for D-specific biotransformations. An analysis of the temperature dependence of the enzyme stereoselectivity can be used for the rational selection of the temperatures where this requirement is fulfilled. In the present work we report some studies on the temperature effects on S_1- and S'_1-enantioselectivity of bovine α-chymotrypsin.

2. Materials and methods

2.1. α-Chymotrypsin

Bovine α-chymotrypsin (E.C. 3.4.21.1) was purchased from Worthington (Freehold, NJ) as recrystallised powder. The purity of the enzyme was determined by isoelectric focusing. The enzyme was practically homogeneous (more than 90% of the active protein was located in one band). The concentration of the active sites, determined by spectrophotometric titration with *N-trans*-cinnamoylimidazole [27] was found to be 84%.

2.2. Chemicals

N^{α}-*t*-Butyloxycarbonyl-L-tyrosine methyl ester, N^{α}-*t*-butyloxycarbonyl-D-tyrosine methyl ester, D-tyrosine methyl ester, L-arginine amide and L-valine amide were purchased from Bachem (Heidelberg). N^{α}-Acetyl-L-tyrosine ethyl ester was obtained from Serva (Heidelberg). L-Tyrosine methyl ester was purchased from Aldrich (Steinheim). N^{α}-Acetyl-L-tyrosine (*N*-Ac-L-Tyr) and D-phenylglycine were obtained from Sigma (Deisenhofen). N^{α}-Acetyl-L-phenylglycine methyl ester, N^{α}-acetyl-D-phenylglycine methyl ester, L- and D-phenylglycine methyl ester were obtained from Röhm (Darmstadt). L-Tyrosine and all other chemicals were of analytical grade and were purchased from Merck (Darmstadt). D-Arginine amide and D-valine amide were synthesised as described [28].

2.3. Kinetic measurements and calculations

In a typical experiment for α-chymotrypsin catalysed hydrolysis of enantiomeric Tyr and Phg derivatives, 1.5 ml substrate solution in phosphate buffer $I = 0.2$ with pH adjusted to 7.5 at each temperature was placed in a screw-cap vial and preincubated at the appropriate temperature. The reaction was started by addition of enzyme solution preincubated for 3 min at the same temperature in the same buffer. The stock solutions of the enzyme had concentrations in the range 2×10^{-8}–1×10^{-3} M. During the enzyme preincubation autolysis was not observed, as determined by active site titration. Reactions were carried out over the 5–45°C range in a water thermostat with temperature maintained within ±0.1°C accuracy. The enzyme concentration in the reaction mixture was from 1.2×10^{-4} to 2×10^{-6} M for the hydrolysis of the D-enantiomers and from 2×10^{-5} to 3.4×10^{-10} M for the hydrolysis of the L-enantiomers. Periodically, aliquots were withdrawn and immediately assayed by HPLC. The elution times were less than 3 min and the injection served as stopping point of the enzyme reaction. The initial rates ($\sim$ 10% substrate exhausting) were determined on the basis of the increase in the concentration of the hydrolysis products (L- and D-Tyr, L- and D-Phg, *N*-Boc-L-Tyr, *N*-Boc-D-Tyr, *N*-Ac-L-Phg and *N*-Ac-D-Phg) as a function of time. Five to six points were measured. The calculations were performed by linear regression analysis using the PlotIT software, version 3.14 (Scientific Programming Enterprises, 1994). The

correlation coefficients were always > 99. The initial rate at each temperature was the average of at least 3 experiments, the standard deviations being less than 10%. Each experiment for α-chymotrypsin catalysed hydrolysis was paralleled by a reference reaction without enzyme for a reading of the spontaneous hydrolysis (at 45°C it can reach 20% of the initial rate of the enzyme catalysed reaction). The rate of the spontaneous hydrolysis was used to correct the input for calculation of the kinetic constants. The values of K_m and k_{cat} for each enantiomer at each temperature were determined on the basis of the dependencies of the initial rates on the substrate concentrations (six to seven concentrations in the range from $0.5 \times K_m$ to $5 \times K_m$). The calculations of K_m and k_{cat} were performed by non-linear regression analysis using the software program Enzfitter, written by R.J. Leatherbarrow and distributed by Elsevier Biosoft (Cambridge 1987).

In a typical experiment for α-chymotrypsin catalysed kinetically controlled synthesis of diastereomeric dipeptides, 1.5 ml reaction mixture containing 10 mM *N*-Ac-TyrOEt and 100 mM nucleophile in bicarbonate buffer $I = 0.2$ with pH adjusted to 9.0 at each temperature was placed in a screw-cap vial and preincubated at the appropriate temperature. The reaction was started by addition of enzyme solution preincubated for 3 min at the same temperature in the same buffer. Similar to the hydrolysis experiments, enzyme autolysis was not observed during the preincubation. Measurements were performed over the 5–45°C range within ± 0.1°C accuracy. The final enzyme concentration in the reaction mixture was (depending on the nucleophile and its concentration) in the range 6×10^{-9}–6.6×10^{-10} M. Periodically, aliquots were withdrawn and immediately analysed by HPLC. The elution times were less than 3.5 min and the injection served as stopping point of the enzyme reaction. Each experiment for α-chymotrypsin catalysed kinetically controlled synthesis was paralleled by a reference experiment without enzyme for a reading of the spontaneous hydrolysis which was used to correct the input for calculation of k_T/k_H. The initial rates of the reactions of transfer (v_T) and hydrolysis (v_H) were determined on the basis of the increase in the concentrations of the condensation, respectively, hydrolysis products as a function of time by linear regression analysis using the PlotIT software (six to seven points were measured). These initial rates were average of several experiments at each temperature. The statistical analysis was the same as for the hydrolytic reactions described above. The standard deviations were less than 10%. The transferase to hydrolase ratios k_T/k_H for each enantiomer at each temperature were calculated using the relation $k_T/k_H = v_T[H_2O]/v_H[N]$ [24].

2.4. HPLC analysis

Products and reactants were identified and analysed using a Bruker LC 22 Solvent Delivery System and LKB Bromma Variable Wavelength Detector with RP-18 or RP-8 5 μm column from Merck, thermostated at 50°C. The substances were quantified using calibration curves for the peak areas obtained with reference compounds of known concentrations and the Bruker Chrom Star software 1994. For quantification of the diastereomeric dipeptides, the calibration curves for *N*-Ac-L-Tyr-OMe were used since for the absorption at 280 nm dominant is the Tyr moiety (the difference of the peak areas for 1 mM *N*-Ac-L-TyrOEt and 1 mM *N*-Ac-L-Tyr is $< 1\%$). The elution system for the separation of *N*-Boc-L-TyrOMe and *N*-Boc-L-Tyr, respectively, *N*-Boc-D-TyrOMe and *N*-Boc-D-Tyr was: methanol/0.003 M KH_2PO_4 pH 4.5 (30 : 70 v/v), flow rate 1.5 ml/min and detection at 225 nm (RP-18 column); for L-TyrOMe and L-Tyr, respectively, D-TyrOMe and D-Tyr: 0.067 M KH_2PO_4 pH 6.0, flow rate 1.5 ml/min and detection at 225 nm (RP-18 column); for Ac-L-PhgOMe and Ac-L-Phg, respectively, Ac-D-PhgOMe and Ac-D-Phg: MeOH/0.067 M KH_2PO_4 pH 6.0 (30/70 v/v), flow rate 2 ml/min, detection at 225 nm (RP-8 column); for *N*-Ac-L-TyrOEt, *N*-Ac-L-Tyr, L-ArgNH_2 and *N*-Ac-L-Tyr-L-ArgNH_2, D-Arg-NH_2 and *N*-Ac-L-Tyr-D-ArgNH_2, L-Val-NH_2 and *N*-Ac-L-Tyr-L-ValNH_2, D-Val-NH_2 and *N*-Ac-L-Tyr-D-ValNH_2: MeOH/0.003 M KH_2PO_4 pH 4.5 (30 : 70 v/v), flow rate 1.5 ml/min, detection at 280 nm (RP-18 column).

3. Results

3.1. S_1-enantioselectivity

The temperature effect on S_1-enantioselectivity of α-chymotrypsin for the hydrolysis of derivatives of

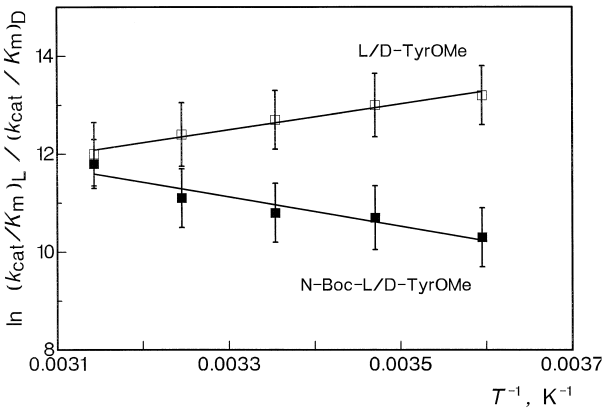

Fig. 2. Arrhenius plots for the ratios of the specificity constants for α-chymotrypsin-catalysed hydrolysis of the enantiomeric pairs L/D-TyrOMe and *N*-Boc-L-TyrOMe/*N*-Boc-D-TyrOMe. Reaction conditions: phosphate buffer pH 7.5, $I = 0.2$. The lines are result of linear regression analysis of the experimental data using the PlotIT software. Error bars indicate the standard deviations of *E*. The plots were used for calculation of enthalpic and entropic differences and the reversal temperatures.

two amino acids – the specific Tyr and the unspecific, non-proteinogenic phenylglycin (Phg) was studied over the 5–45°C range. The K_m values for the hydrolysis of the enantiomeric methyl esters of Tyr with free and protected amino groups N^{α}-*t*-butyloxycarbonyl-L-TyrOMe (*N*-Boc-L-TyrOMe), N^{α}-*t*-butyloxycarbonyl-D-TyrOMe (*N*-Boc-D-TyrOMe), L-TyrOMe and D-TyrOMe are practically independent of the temperature. The catalytic (k_{cat}) and specificity (k_{cat}/K_m) constants increase with the temperature, but the slopes for the enantiomers are not identical (data not shown). From the Arrhenius plots of the enantiomeric ratios (Fig. 2) T_r and the differences in the enthalpic and entropic terms were determined using Eqs. (2) and (5). They are presented in Table 1. The ''reversal'' temperature for the enantiomeric *N*-Boc-L-TyrOMe and *N*-Boc-D-TyrOMe was estimated to be < -100°C and in the studied temperature interval, the stereospecificity increases with increasing temperature. Both $\Delta\Delta H$ and $\Delta\Delta S$ are positive. For the unprotected L- and D-TyrOMe, $\Delta\Delta H$ and $\Delta\Delta S$ are negative, T_r is much above the studied temperature interval (Table 1) and the enantiomeric ratio decreases with increasing temperature (Fig. 2).

The temperature effects on the kinetic parameters for the hydrolysis of the unspecific enantiomeric Phg derivatives N^{α}-acetyl-L-PhgOMe (*N*-Ac-L-PhgOMe), N^{α}-acetyl-D-PhgOMe (*N*-Ac-D-PhgOMe), L- and D-PhgOMe reveal that in this case the discrimination is influenced by the temperature in both K_m and k_{cat} (data not shown). From the Arrhenius plots for the ratios of the specificity constants for both enantiomeric pairs (Fig. 3) and Eqs. (2) and (5) T_r and the values of $\Delta\Delta H$ and $\Delta\Delta S$ which are negative were determined (Table 1). For L- and D-PhgOMe, T_r is within and for Ac-L-PhgOMe and Ac-D-PhgOMe is above the range 5–45°C and the enantiomeric ratios decrease with increasing temperature. For L- and D-PhgOMe hydrolysis, stereochemical reversal of the reaction occurs at 35°C. Below this temperature L-PhgOMe hydrolysis predominates, above this temperature the hydrolysis of D-PhgOMe is favoured.

3.2. S'_1-enantioselectivity

The temperature dependence of S'_1-enantioselectivity of α-chymotrypsin was studied for the kinetically controlled peptide synthesis using N^{α}-acetyl-L-TyrOEt (Ac-L-Tyr-OEt) as an acyl donor and the

Table 1
Enthalpic and entropic differences for α-chymotrypsin catalysed hydrolysis of enantiomeric methyl esters of Tyr and Phg with free and protected amino groups [a]

Substrate	$\Delta\Delta H$ (kJ mol^{-1})	$\Delta\Delta S$ (J mol^{-1} deg^{-1})	T_r (°C)
L and D *N*-Boc-TyrOMe	25 ± 3	174 ± 20	−129 ± 16
L- and D-TyrOMe	−22 ± 3	−31 ± 4	436 ± 52
L and D *N*-Ac-PhgOMe	−36 ± 5	−69 ± 8	248 ± 30
L- and D-PhgOMe	−37 ± 5	−120 ± 16	35 ± 5

[a] Derived by fitting of the curves in Figs. 2 and 3 to Eq. (2): $\Delta\Delta H$ were determined from the slopes of the curves; T_r were determined from the intercepts of the curves with the abscissa; $\Delta\Delta S$ were determined from ordinate intercepts or using Eq. (5). The statistical analysis of the derived thermodynamic parameters was performed as described in H.D. Young, Statistical Treatment of Experimental Data, McGraw-Hill, 1962.

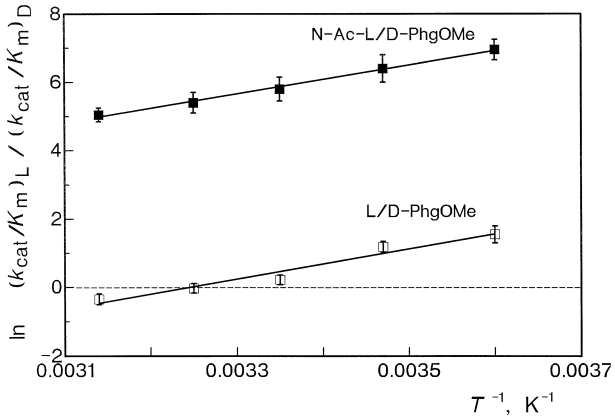

Fig. 3. Arrhenius plots for the ratios of the specificity constants for α-chymotrypsin-catalysed hydrolysis of the enantiomeric pairs L/D-PhgOMe and *N*-Ac-L-PhgOMe/*N*-Ac-D-PhgOMe. Reaction conditions: see Fig. 2. The lines are results of linear regression analysis of the experimental data using PlotIT software. Error bars indicate the standard deviations of E.

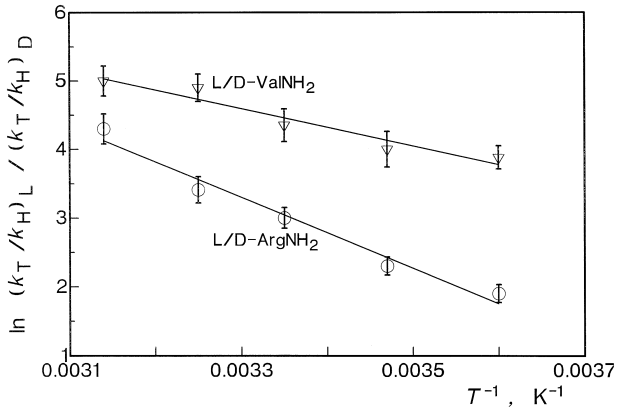

Fig. 4. Arrhenius plots for the ratios of the transferase to hydrolase ratios k_T/k_H for α-chymotrypsin catalysed acyl transfer from *N*-Ac-L-TyrOEt to the enantiomeric nucleophiles L/D-$ArgNH_2$ and L/D-$ValNH_2$. Reaction conditions: bicarbonate buffer pH 9.0, $I = 0.2$; *N*-Ac-L-TyrOEt concentration 10 mM, nucleophile concentration 100 mM, enzyme concentration 6.6×10^{-10}–6.6×10^{-9} M. The lines are result of linear regression analysis of the experimental data as described for Figs. 2 and 3. Error bars indicate the standard deviations of E'.

enantiomeric L- and D-$ArgNH_2$ and L- and D-$ValNH_2$ as nucleophiles in the interval 5–45°C. The temperature influences the transferase to hydrolase ratios for the enantiomers of $ArgNH_2$ and $ValNH_2$ in a different way. k_T/k_H for the L-enantiomers increases with increasing temperature, while k_T/k_H for the D-enantiomers decreases (data not shown). The ''reversal'' temperatures determined from the Arrhenius plots (Fig. 4) are below the studied temperature range (Table 2) and S'_1-enantiospecificity increases with increasing temperature. The values of $\Delta\Delta H'$ and $\Delta\Delta S'$ for the kinetically controlled acyl transfer to the enantiomeric L- and D-$ArgNH_2$ and L- and D-$ValNH_2$ determined from the Arrhenius plots in Fig. 4 are presented in Table 2. They are positive.

4. Discussion

4.1. S_1-enantioselectivity

Under entropic control, the positive activation enthalpy and entropy differences for the hydrolysis of the pair *N*-Boc-L/D-TyrOMe imply increasing im-

Table 2
Enthalpic and entropic differences for α-chymotrypsin catalysed kinetically controlled synthesis of the diastereomeric dipeptides *N*-Ac-L-Tyr-L-$ArgNH_2$/*N*-Ac-L-Tyr-D-$ArgNH_2$ and *N*-Ac-L-Tyr-L-$ValNH_2$/*N*-Ac-L-Tyr-D-$ValNH_2$ [a]

Nucleophile	$\Delta\Delta H'$ (kJ mol^{-1})	$\Delta\Delta S'$ (J mol^{-1} deg^{-1})	T_r (°C)
L- and D-$ArgNH_2$	43 ± 5	169 ± 17	-19 ± 3
L- and D-$ValNH_2$	23 ± 2	113 ± 10	-70 ± 8

[a] Derived by fitting of the curves in Fig. 4 to Eq. (2): $\Delta\Delta H'$ were determined from the slopes of the curves; T_r were determined from the intercepts of the curves with the abscissa; $\Delta\Delta S'$ were determined from ordinate intercepts or using Eq. (5). The statistical analysis was the same as for the thermodynamic parameters in Table 1.

pact of the entropic term with increasing temperature (Eq. (2)). This results in higher stereoselectivity at higher temperature (Fig. 2). Since k_{cat}/K_m is an apparent second order rate constant, the observed enthalpic and entropic differences reflect both differences in binding and (or) catalysis. The structural basis of α-chymotrypsin enantioselectivity is one of the most investigated [29,30]. Domination of the entropic term in the activation energy difference for the hydrolysis of *N*-Boc-L/D-TyrOMe could be partly due to the water release by the substrate binding. The productive binding of a specific *N*-acyl-L-amino acid substrate to α-chymotrypsin active site results in a release of ordered water molecules from the hydrophobic pocket [31]. In order to be reactive, the D-enantiomer will bind ''incorrectly'' [32]. As a result fewer water molecules will be excluded and the bulky acylamino moiety (BocNH-) in the enantiomeric substrates could exert different degrees of steric hindrance by enzyme acylation and deacylation [33]. The positive $\Delta\Delta H(\Delta\Delta H^{\#} - \Delta\Delta H_b)$ implies that in this case the substrate binding has a larger influence on the stereoselectivity than the transition state stabilisation [34,35]. For the unprotected L- and D-TyrOMe, the temperature influence is reversed. $\Delta\Delta H$ and $\Delta\Delta S$ are negative and the enantiomeric ratio decreases with increasing temperature (Fig. 2).The negative $\Delta\Delta H$ for the hydrolysis of L/D-TyrOMe reflects more favourable interactions between the L-enantiomer and the enzyme active site in the transition state. L-TyrOMe without bulky amino group protection could realize most of the favourable hydrogen bonds and Van der Waals contacts responsible for the stabilisation of the transition state. For a productive binding of the D-enantiomer, part of these interactions are probably not realized and its transition state is less rigid and has less rotational restrictions, i.e. $\Delta\Delta S$ is negative. Here the temperature dependence of the unprotected amino group pK_a, respectively, the strength of the hydrogen bonding or induced charge–charge interactions should be also considered.

Under enthalpic control, the negative values of $\Delta\Delta H$ and $\Delta\Delta S$ for Ac-L/D-PhgOMe hydrolysis determine decreasing stereoselectivity with increasing temperature. For the unprotected L/D-PhgOMe, the temperature influence is similar, but T_r is near the temperature optimum of the enzyme (Fig. 3). For L/D-PhgOMe, the discrimination is very weak in K_m and k_{cat} terms (data not shown). The negative $\Delta\Delta H$ for the hydrolysis of L and D AcPhgOMe indicates that in this case the interactions in the transition state have larger influence on the stereoselectivity than the binding of the substrate. The structure of the unspecific Ac-PhgOMe differs from the aminoacylated specific ester substrates of α-chymotrypsin by only one methylene group, but it seems to be crucial for the productive binding and stabilisation of the transition state of the hydrolytic reaction (the K_m values for the hydrolysis of Ac-L-PheOMe and Ac-L-PhgOMe are the same order of magnitude, but k_{cat} for the Phe derivative is two orders of magnitude higher, data not shown). Thus, this is an example where the binding interactions do not contribute to the stabilisation of the transition state. For a binding of the D-enantiomer in a reactive way, these interactions would be disrupted much more or even not realized, which would result in a higher conformational freedom and (or) exposure to the solvent (negative $\Delta\Delta H$ and $\Delta\Delta S$). The removal of the amino group protection turns PhgOMe into a more unspecific substrate, which even in L-configuration, could not properly be accommodated in the enzyme active site. Therefore, in this case of nonproductive binding the discrimination between the enantiomers for a proper alignment to the active Ser-195 disappears. This results in stereochemical reversal of the reaction at 35°C (T_r) (Fig. 3).

4.2. S'_1-enantioselectivity

The nucleophile (S'_1) enantioselectivity of α-chymotrypsin is dependent on the correct orientation of the nucleophile α-amino group to the $>C=O$ group of the acyl-enzyme and on the interaction of the amino acid side chain in P'_1 position with the enzyme binding subsite S'_1 [36–39]. For the studied nucleophiles L/D-ArgNH_2 and L/D-ValNH_2, $\Delta\Delta H$ and $\Delta\Delta S$ are positive. In the range 5–45°C, the stereochemical course of the synthetic reaction is dominated by the entropy difference and the stereochemical purity of the diastereomeric dipeptides increases with increasing temperature. The small number of contacts between the enzyme and the amino acid amide [40] allows a large conformational freedom of the nucleophile during its attack. This could

explain the positive entropy difference and its domination in enantioselectivity. The D-nucleophile possibly realizes similar hydrogen bonding or electrostatic interactions, but in ''incorrect'' position. Thus, the proper alignment of the NH_2 group for the nucleophilic attack to the acyl-enzyme could then be paid by loss of conformational freedom.

The positive $\Delta\Delta H$ implies that the binding interactions in acyl-enzyme–nucleophile complex have a larger influence on L/D discrimination than the interactions in the transition state of deacylation. All this is difficult to discuss in exact molecular terms because the energy of interaction can be partially consumed in a conformational change of the protein globule, which will be different for the L- and D-nucleophile.

5. Conclusions

Quantitative determinations with α-chymotrypsin showed that E can differ by orders of magnitude depending on the substrate. By variation of the temperature in the range 5–45°C, the enantiomeric ratios for the studied α-chymotrypsin catalysed hydrolytic and synthetic reactions were modulated 3–15 fold. The optimal purity may require either increasing or decreasing of the reaction temperature. For the hydrolytic reactions, both enthalpic and entropic control of the discrimination were observed, respectively, decreasing or increasing of S_1-enantioselectivity with temperature was possible. For one unspecific substrate, a temperature dependent stereochemical reversal was observed near the temperature optimum of the enzyme. For the kinetically controlled synthetic reactions, the entropy difference dominates and only increasing of S_1'-enantioselectivity with increasing temperature was observed. Most of the studied substrates possess polar groups responsible for ionic interactions and hydrogen bonding and strong enthalpy difference domination could be expected [20]. Nevertheless, for these substrates the enantioselectivity was temperature dependent. For the reactions where E increases with increasing temperature, the values of $\Delta\Delta H$ and $\Delta\Delta S$ are > 0. Positive $\Delta\Delta H$ implies that in these cases the binding of the substrate (nucleophile) has a larger influence on the stereoselectivity than the interactions in the transition state or that ground-state interactions could also contribute to the stabilisation of the transition states in enzyme-catalysed processes.

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