Specificities of Enzymes "Corrected for Solvation" Depend on the Choice of the Standard State

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Abstract: The observed specificity of enzymes is affected by the reaction medium and can be, at least partly, explained by a change in substrate solvation. By using a transfer free energy method, enzyme kinetics in an organic solvent can be corrected to a chosen standard state. The choice of standard state does not affect conclusions about a single substrate. However, when two or more substrates are compared, the "corrected specificity" becomes a function of the standard state. To illustrate this, we have studied the esterification of sulcatol and several saturated fatty acids catalyzed by *Candida rugosa* lipase. The observations in toluene (based on V_m/K_m) show a preference for C4, C8, C10, and C12 fatty acids. Correction to the pure liquid standard state suggests more or less the same specificity. But corrections to the gas phase or dilute aqueous standard states would suggest a strong preference for longer-chain fatty acids. Hence, such corrected specificities should be used and interpreted only with great care.

Introduction

The observed specificity of enzymes often changes substantially when they are used in organic media.[1] An important cause is the different solvation of the substrates as the medium is changed. These solvation effects may be corrected for on the basis of the free energy of transfer or thermodynamic activity of the substrate.[2-5] Conclusions on the relative effects of different solvents with a single substrate are independent of the choice of the standard state. Comparisons have also been made of corrected parameters for two or more different substrates.[2c,3,4] In this paper we show how the results of such a comparison will depend on the arbitrary choice of the standard state. Thus, such calculated "corrected substrate specificities" should be used and interpreted only with caution.

Results and Discussion

In order to study how substrate solvation affects the specificity of enzymes in organic media, we have chosen the esterification of several saturated fatty acids with sulcatol, catalyzed by *Candida rugosa* lipase, as a model reaction. The kinetic parameters V_m/K_m and K_m^{app} are shown in Figure 1. K_m^{app} increases slightly with fatty acid chain length; however, the effect of chain length is most pronounced on V_m/K_m. For long-chain fatty acids (C14 and higher), V_m/K_m is low, while V_m/K_m is high for fatty acids with a chain length up to 12. Hexanoic acid forms an exception with a very low V_m/K_m value, which has been observed before for this lipase.[3,6]

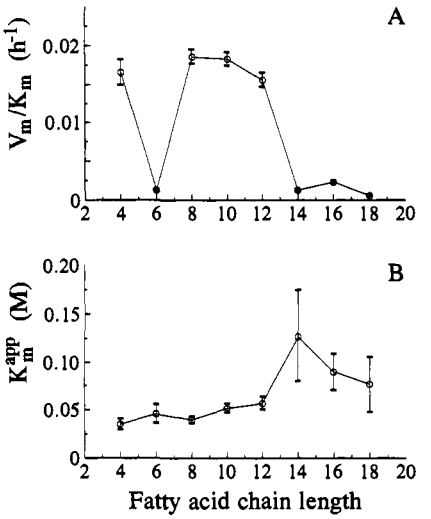

Figure 1. Dependence of V_m/K_m (A) and K_m (B) on fatty acid chain length for the *Candida rugosa* lipase-catalyzed esterification of sulcatol ((±)-6-methyl-5-hepten-2-ol) and saturated fatty acids in toluene at a_w = 0.76 (see the Experimental Section for details). The error bars represent the 95% confidence deviations.

To correct for solvation effects, enzyme kinetics in an organic solvent are often related to a chosen reference or standard state[7] by using a transfer free energy method.[2-5] This method involves a thermodynamic cycle from which the activation energy in the standard state ($\Delta G^{\ddagger ref}$) can be calculated:

$$\Delta G^{\ddagger ref} = \Delta G^{\ddagger solv} - \Delta G^{t}_{E} - \Delta G^{t}_{S} + \Delta G^{t}_{ES} \qquad (1)$$

The activation energy in the solvent or standard state is related to the specificity constant (k_{cat}/K_m):[8,9]

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(6) Rangheard, M.-S.; Langrand, G.; Triantaphylides, C.; Baratti, J. *Biochim. Biophys. Acta* **1989**, *1004*, 20–28.

(7) The standard state is sometimes implicitely chosen; for example, if activity coefficients are calculated by using the UNIFAC method, the standard state is the pure (supercooled) liquid.

(8) Fersht, A. *Enzyme structure and mechanism*, 2nd ed.; Freeman: New York, 1985. Kraut, J. *Science* **1988**, *242*, 533–540.

(9) The same reasoning is valid to relate the binding energy (ΔG_b) to the dissociation constant of the enzyme–substrate complex (K_S) or approximately K_m.

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$$\Delta G^{\ddagger} = -RT \ln \frac{(k_{cat}/K_m)h}{k_B T} \quad (2)$$

Here, h is Planck's constant, k_B is Boltzmann's constant, and T is the absolute temperature. In eq 1, ΔG^t_E, ΔG^t_S, and ΔG^t_{ES} represent the transfer of the enzyme, substrate, and enzyme–substrate complex from an organic solvent to an arbitrary standard state. The transfer free energy of the substrate is related to

$$\Delta G^t_S = -RT \ln (f^\circ_{solv}/f^\circ_{ref}) \quad (3)$$

Here, f°_{solv} refers to the fugacity in an infinitely dilute organic solvent and f°_{ref} is used for the fugacity in the standard state. Possible choices of the standard state include the pure liquid substrate, the vapor phase, and the infinitely dilute aqueous solution. The various standard states, fugacities, and transfer free energies are schematically shown in Figure 2.

If we now consider two different substrates, we can write for each an equation analogous to eq 1. Subtracting these two equations then gives

$$\Delta\Delta G^{\ddagger ref}_{(1\to2)} - \Delta\Delta G^t_{ES(1\to2)} = \Delta\Delta G^{\ddagger solv}_{(1\to2)} - \Delta\Delta G^t_{S(1\to2)} \quad (4)$$

Substituting eqs 2 and 3 into eq 4 results in

$$-RT \ln \frac{(k_{cat}K_m)_{1,ref}}{(k_{cat}/K_m)_{2,ref}} - \Delta\Delta G^t_{ES(1\to2)} = -RT \ln \frac{(k_{cat}/K_m)_1}{(k_{cat}/K_m)_2} + RT \ln \frac{(f^\circ_{solv}/f^\circ_{ref})_1}{(f^\circ_{solv}/f^\circ_{ref})_2} \quad (5)$$

$\Delta\Delta G^{\ddagger solv}$ and $\Delta\Delta G^t_{S(1-2)}$ can be calculated from k_{cat}/K_m[18] and the fugacities of the substrates, respectively. ΔG^t_{ES} has been shown to be constant for different substrates in peroxidase catalysis in organic media.[2c] If this is general,[19] the term $\Delta\Delta G^t_{ES(1-2)}$ is zero and $\Delta\Delta G^{\ddagger ref}$ can be calculated from the measured k_{cat}/K_m in an organic solvent and the transfer free energies of the substrates (eq 5). The latter value will be dependent on the choice of the standard state because changing the standard state will alter f°_{ref} values and usually also their ratio. This means that the corrected value of $(k_{cat}/K_m)_1/(k_{cat}/K_m)_2$ is a function of the standard state chosen.

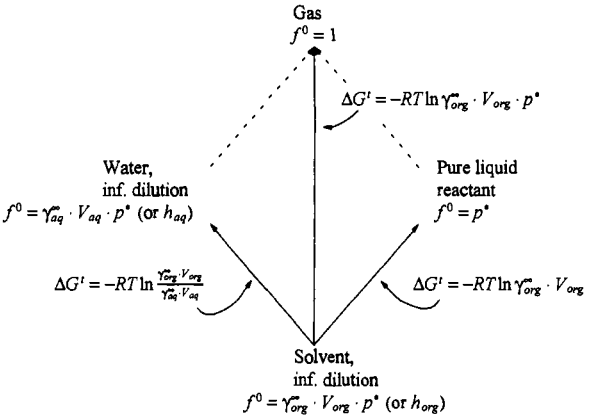

Figure 2. Possible choices of standard states and the corresponding fugacities and transfer free energies. Activity coefficients in infinitely dilute solutions (γ^∞_{org} or γ^∞_{aq}) can be calculated using the UNIFAC group contribution method.[10] For the calculations in this paper, a data set for vapor–liquid equilibria was used.[10] For the calculations in this paper, a data set for vapor–liquid equilibria was used.[11,12] The γ values are based on a mole fraction scale. Since k_{cat}/K_m or K_m usually incorporate molar concentration units, the mole fraction based γ values are converted to molar concentration based γ values by multiplying by the molar volume (V_{org} or V_{aq}). Vapor pressures (p^*) can be obtained from literature data[13] or calculated with a group-contribution model, which is partly based on the UNIFAC method for vapor–liquid equilibria.[14] Several Henry's law constants in water (h_{aq}) or in organic solvent (h_{org})[15] are known.[16,17] Alternatively, group-contribution methods are available to estimate Henry's constants.[16,17]

Corrected Specificities for Different Standard States

The dependence of the corrected specificity on the choice of the standard state is illustrated in Figure 3A. This shows the observed fatty acid specificity of *Candida rugosa* lipase in toluene and the corrected specificities with the pure liquid substrate, a dilute aqueous solution, and the gas phase as the standard states. The higher the value of $\Delta\Delta G^{\ddagger ref}$, the less the fatty acid is favored compared to butanoic acid. As discussed before, the enzyme shows a preference for C4, C8, C10, and C12 fatty acids in toluene. The specificity is more or less the same when corrected to the pure liquid substrate standard state. However, a strong preference for long-chain fatty acids is suggested using the dilute aqueous solution or the gas phase standard state ($\Delta\Delta G^{\ddagger ref}$ has a large negative value). The observed and corrected binding energies (compared to those of butanoic acid) are shown in Figure 3B. In toluene, the binding is slightly stronger for short-chain fatty acids. Almost no differences are detected if the binding is corrected to the pure liquid standard state, and the binding is very strong for the long-chain fatty acids in the case of corrections using the dilute aqueous solution or the gas phase as the standard state.

A recent report describes correction of specificity constants for lipase-catalyzed esterification of several 2-hydroxy acids with *n*-butanol using activity coefficients estimated with the UNIFAC group contribution method.[3] This gives values referred to the pure liquid standard state. In Figure 4a, we have plotted experimental $\Delta\Delta G^{\ddagger solv}$ values for the hydroxy acids,[3] fatty acid ethyl esters,[6] and fatty acid vinyl esters.[3] Corrections for solvation effects with the pure liquid substrate and a dilute aqueous solution

(10) Fredenslund, A.; Gmehling, J.; Rasmussen, P. *Vapor-liquid equilibria using UNIFAC*; Elsevier: New York, 1977.

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(12) For calculations in aqueous–organic two-phase systems, the UNIFAC parameter set for liquid–liquid equilibria might be more useful (Magnussen, T.; Rasmussen, P.; Fredenslund, A. *Ind. Eng. Chem. Process Des. Dev.* **1981**, *20*, 331–339).

(13) Boublik, T.; Fried, V.; Hala, E. *The vapour pressures of pure substances*, 2nd ed.; Elsevier: Amsterdam, 1984. D'Ans-Lax. *Taschenbuch fur chemiker und physiker*, 3rd ed.; Springer-Verlag: Berlin, 1967; Vol. 1, pp 912–947.

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(15) The Henry's law constant, h_{org}, is usually the same in different aliphatic hydrocarbons (ref 16, p 92).

(16) Grant, D. J. W.; Higuchi, T. *Solubility behavior of organic compounds*; Wiley: New York, 1990.

(17) Abraham, M. H. *J. Chem. Soc., Faraday Trans. 1* **1984**, *80*, 153–181.

(18) By comparing two substrates, the enzyme concentration cancels out and V_m/K_m can be used (if the same enzyme concentration is used for both substrates).

(19) It has recently been suggested that $\Delta\Delta G^t_{ES(1-2)}$ is not zero for a subtilisin-catalyzed transesterification.[4b]

(20) The *y*-axis actually shows the calculated value of $\Delta\Delta G^{ref} - \Delta\Delta G^t_{ES}$ (eq 5) with the latter often expected to be zero.

(21) UNIFAC calculations may be made using the full composition of the organic phase. A high alcohol concentration will have an enormous effect on the value of the activity coefficient. However, this effect is partly cancelled out by comparing two substrates in the same reaction mixture. Therefore, the relative estimates will be similar to those using the Henry's law constant, which is for the pure organic solvent.

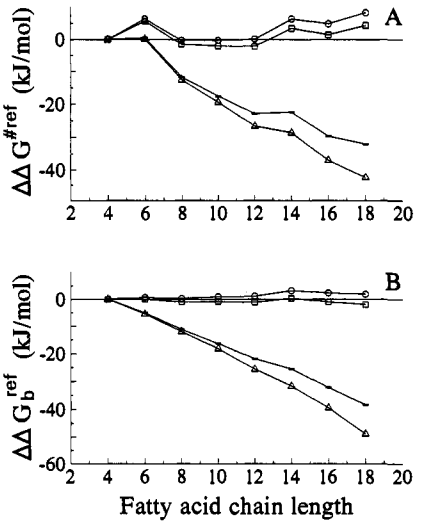

Figure 3. Activation energy (A) and binding energy (B) of the *Candida rugosa* lipase-catalyzed reaction of sulcatol with various fatty acids observed in toluene (○) and corrected to the pure liquid fatty acid (□), the vapor phase (△), and dilute aqueous solution (–) standard states. Equation 5 is used to calculate[20] $\Delta\Delta G^{\#ref}$ and $\Delta\Delta G_b^{ref}$. $\Delta\Delta G^{\dagger}_{S(1-2)}$ is calculated by using the standard states defined in Figure 2. The vapor pressures are calculated by using a group-contribution model.[14] The activity coefficients in infinitely dilute solutions (γ_{aq}^{∞} or γ_{org}^{∞}) are calculated by using the UNIFAC group-contribution method[10] and the data set for vapor–liquid equilibria.[11] In these calculations the effects of all components of the organic phase are considered.[21]

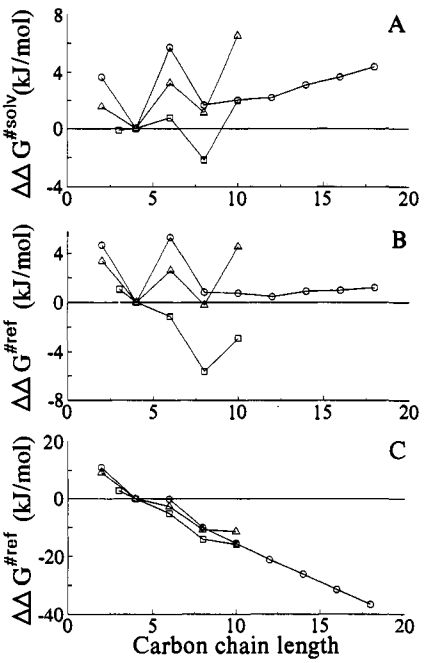

Figure 4. Specificity of *Candida rugosa* lipase for various 2-hydroxy acids (□), vinyl esters (△), and ethyl esters (○). Part A shows the observed specificity, and in parts B and C, the specificities are corrected to the pure liquid substrate and dilute aqueous solution standard states, respectively. The k_{cat}/K_m values for the 2-hydroxy acids and vinyl ester were obtained by (trans)esterification with *n*-butanol in cyclohexane.[3] The values for the ethyl ester were for transesterification with *n*-propanol in pentane.[6] Equation 5 is used to calculate[20] $\Delta\Delta G^{\#ref}$, and $\Delta\Delta G^{\dagger}_{S(1-2)}$ is calculated by using the standard states as defined in Figure 2. The vapor pressures are calculated by using a group-contribution model.[14] The activity coefficients in infinitely dilute solutions (γ_{aq}^{∞} or γ_{org}^{∞}) are calculated by using the UNIFAC group-contribution method[10] and the data set for vapor–liquid equilibrium.[11] In these calculations the effects of all components of the organic phase are considered.[21]

as the standard states are shown in Figure 4B,C. If we consider the fatty acid ethyl ester in pentane, Figure 4A shows that the C2 and C6 esters are poor substrates. The esters with a chain length above eight are poorer substrates if the chain length increases. If the specificity is corrected to the pure substrate standard state (Figure 4B), the C2 and C6 esters still appear to be poor substrates; however, there is no difference in the corrected specificities of the esters with a chain length above eight. If the dilute aqueous solution standard state is used (Figure 4C), the substrates appear to be better with increasing chain length. The graphs are slightly different for the hydroxy acids and vinyl esters. Both Figures 3 and 4 clearly show that the corrected substrate specificity is dependent on the choice of the standard state and on the type of substrate.

Advantages of Different Choices of the Standard State

To correct measured parameters, solvation must be measured or predicted in both the test solvent and the chosen standard state. Therefore, in the preferred standard state, data on substrate solvation should be available in the literature or easily measured or predicted. It is also useful to be able to measure enzymatic behavior in the standard state itself, for comparison with corrected parameters. In this section, we will discuss some possible standard states in these terms.

(i) In the pure liquid reactant standard state, the enzymatic reaction cannot be measured, since most reactions of interest involve two substrates. Data on substrate solvation (e.g., solubility data) are available if the substrate is a liquid; however, for solid substrates, the standard state is not physically attainable (usually the hypothetical supercooled liquid is used). The main advantage of this standard state is that good predictions can be made with group contribution methods, such as the UNIFAC method.

(ii) In the infinitely dilute aqueous solution, there are a lot of data available on enzymatic reactions, although the reaction of interest might not be possible in aqueous solution (e.g., esterification reactions). Data on substrate solvation (e.g., partition coefficients) are available or can be measured. However, predictions are usually hard because of the complex solvation in aqueous solutions.

(iii) For organic molecules, an infinitely dilute alkane solution has been recommended as a standard state.[16] This standard state is most similar to the actual reaction system. Data on enzymatic reactions are available or can easily be measured, although some substrates might be very insoluble in alkanes. Although there might not be too much solvation data available, predictions may be relatively easy because solvation is simple.

(iv) In the gas phase standard state, enzymatic data might be measured, although this is very dependent on substrate volatility. Vapor pressures over the reaction mixture might be measurable. Vapor pressures of pure substrates are often available or can be predicted with group contribution models.[14] In this case, solvation can be related to the gas phase via the pure liquid reactant standard state. In the gas phase there is no solvation, in contrast to the other standard states where solute–solvent or solute–solute interactions have to be considered. Therefore, $\Delta\Delta G^{\#gas}$ values reflect the total direct interaction of enzyme and substrate.

From the above points, we conclude that the pure liquid reactant standard state is less appropriate. We can see reasons for choosing each of the others, although we have a slight overall preference for the gas phase standard state.

Conclusions

In studying enzyme kinetics in organic media, it is important to correct measured values of k_{cat}/K_m and K_m for solvation effects. In comparing a certain substrate and enzyme in different organic solvents, the conclusions are independent of the choice of the standard state. However, if two or more substrates are compared, for example in studying enzyme specificity, the "corrected specificity" becomes a function of the standard state. This implies that studying enzyme specificity cannot be based solely on

calculations or literature data and experimental data on every substrate are necessary in at least one solvent.

Experimental Section

Materials. *Candida rugosa* lipase was obtained from Meito-Sangyo, Nagoya, Japan (OF360) and immobilized by adsorption on Accurel EP100 macroporous polypropylene (Enka, Obernberg, Germany).[22] All chemicals were of 99% or higher purity.

Kinetic Measurements. The organic phase and the immobilized lipase were pre-equilibrated to $a_w = 0.76$ using a saturated NaCl solution.[23] It is important to measure initial rates at the same water activity, since water activity is known to affect the kinetic parameters.[24] In a typical experiment, 5 mL of the pre-equilibrated reaction mixture, containing 0.1 M sulcatol ((±)-6-methyl-5-hepten-2-ol), 0.01–0.1 M fatty acid, and an alkane as the internal standard, was added to 50 mg of the immobilized lipase (0.1 g of crude lipase/g of support). The reaction mixture was shaken at 170 min^{-1} at 22 °C. Samples were taken at regular time intervals, and the ester was analyzed by GLC (Perkin-Elmer) with a 2 m × 3 mm internal diameter column packed with 3% OV-1 on Chromosorb WHP. The carrier gas was N_2 (30 mL/min), and a flame ionization detector was used. The operation temperature was dependent on the fatty acid chain length and increased from 120 °C for the butyl ester to 250 °C for the stearyl ester. Although only the esters were analyzed, derivatization with BSTFA (Bis(trimethylsilyl)trifluoroacetamide, Aldrich) was necessary, for samples containing myristic acid, palmitic acid, and stearic acid, due to tailing of the fatty acid peaks. With the other fatty acids, derivatization was not necessary. The initial reaction rates were determined by linear regression and 6–8 data points were usually collected.

Kinetic Calculations. The initial reaction rates (at eight different substrate concentrations) were fitted to the Michaelis–Menten equation using nonlinear least squares regression (Levenberg–Marquadt method). This two-substrate reaction follows ping-pong kinetics.[24b] Our experiments were carried out at a constant sulcatol concentration, and therefore, the fitted K_m is an apparent value while V_m/K_m is the true value for the fatty acid in the ping-pong equation.

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